Manipulation of the mare's estrous cycle using follicular ablation and effects of maternal dietary omega-3 supplementation on reproductive traits and markers of foal bone metabolism

by

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B.S., Clemson University, 2008
M.S., Clemson University, 2010

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Animal Sciences and Industry
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Abstract

Two experiments were conducted to determine the practicality of utilizing follicular ablation (FA) in mares. Experiment 1 investigated the efficacy of FA as a technique for ovulation synchronization. Twenty non-pregnant mares were assigned to the ablation (FA) or P+E (progesterone + estradiol) treatment (n = 10/treatment). In the FA treatment, follicles > 10 mm were ablated (d 1) and mares were administered $PGF_{2\alpha}$ on d 5. Mares received hCG d 11 and ovaries were monitored via transrectal ultrasound until ovulation. The P+E mares received 150 mg P + 10 mg E for 10 d with concurrent $PGF_{2\alpha}$ administration d 10. Ultrasound monitoring began on d 15 continuing until ovulation. On d 18, P+E mares received hCG. Interval from initiation of treatments to ovulation and interval from hCG administration to ovulation was reduced ($P < 0.01$) in response to FA. Ablation may be an acceptable, non-steroidal alternative for synchronization of ovulation and has the ability to shorten the interval from treatment to ovulation.

The objective of Experiment 2 was to determine if FA could prolong the postpartum interval to ovulation. Eighteen postpartum mares were assigned to receive FA (n = 10) or be untreated controls (CON, n = 8). In FA mares, follicles > 10 mm were ablated on d 6 postpartum. Mares were administered $PGF_{2\alpha}$ on d 11 and monitored via transrectal ultrasound until ovulation. When a follicle ≥ 35 mm was detected, mares received hCG. The CON mares were evaluated via ultrasound beginning on d 4 postpartum until a follicle ≥ 35 mm was detected. Mares were then administered hCG and ovaries were monitored until ovulation. Compared with controls, ablation prolonged ($P < 0.01$) the interval from foaling to ovulation and could be utilized to optimize the timing of breeding to improve postpartum conception rates.

Existing research is limited on foal bone development in response to peri-partum maternal supplementation of n-3 fatty acids. Experiment 3 investigated the effect of maternal n-3 fatty acid supplementation on neonatal bone metabolism and mare reproductive traits. Seventeen pregnant stock-type mares were assigned to either of two treatment diets: Control (CON; n = 8, concentrate with no fat supplementation) or fat-supplemented (FS; n = 9, concentrate plus Gromega™, which provided 13.475 g of eicosapentaenoic acid (EPA) and 11.162 g of docosahexaenoic acid (DHA) per day). Treatment began 8 wk before expected foaling date. Blood samples were collected every other week throughout the trial and additional sampling occurred once a follicle > 30 mm was first detected post-foaling and continued until d 5 post-ovulation. Serum was analyzed for progesterone (P4) and insulin-like growth factor-1 (IGF-1). Follicular activity was monitored via transrectal ultrasound from d 4 postpartum until ovulation. Foal blood collection occurred weekly for 8 wk. At 2 and 4 wk of age, synovial fluid (SF) was collected and submitted for cytology. Plasma and SF were evaluated for markers of bone metabolism. Plasma DHA and EPA were increased ($P < 0.05$) in the FS mares and their foals. No differences ($P > 0.05$) were detected in gestation length, concentrations of IGF-1 or P4, or the postpartum interval to ovulation. In addition, no differences were detected in foal plasma metabolites, SF prostaglandin E_2, or osteocalcin. Compared with CON, SF carboxy-terminal cross-linked telopeptide of type 1 collagen (ICTP) was greater ($P < 0.05$) in the FS treatment at wk 4 and SF total protein was greater ($P < 0.05$) at both time points in the FS treatment. A correlation was detected ($P < 0.05$) between synovial and plasma ICTP concentrations ($r = 0.49$). Maternal n-3 supplementation did not affect mare reproductive traits and had a minimal effect on foal bone metabolism in healthy foals fed to meet their requirements.

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Dedication

This dissertation is dedicated to my family, including my husband Justin, daughters Ryleigh and Gentry, and in loving memory of my mother-in-law, Elizabeth Buist 8/2/1956-3/22/2014.

Chapter 1 - Review of Current Literature for Experiments 1 and 2

Reproductive Hormones in the Cycling Mare

Many different hormones from a variety of sources are responsible for control of the mare's reproductive cycle. Control of the estrous cycle in the mare is a concerted effort of the intricate hypothalamic-pituitary-gonadal axis (Senger, 2005). The hypothalamus, pituitary and gonads are involved in the regulation of many bodily functions, but their primary collective focus is the complex control of reproductive function. These separate endocrine glands act as a single unit through the associated negative and positive feedback effects connected with the various hormones they produce. The hypothalamus is the key regulator of this axis and serves as a link between the nervous and endocrine systems. The hypothalamus releases neurohormones that act on the pituitary that induce release of gonadotropins that target specific sites on the gonads. Stimulation of gonads results in secretion of hormones that then positively or negatively affect hormone production from the hypothalamus and pituitary (Senger, 2005). The following review will cover the primary hormones involved in the regulation of the estrous cycle of the mare.

Gonadotropin-Releasing Hormone

Gonadotropin-releasing hormone (GnRH) is a neuro-decapeptide highly conserved across species (Ginther, 1992). It is produced by neurosecretory cells located in the hypothalamus (Senger, 2005). The pineal gland acts as the regulator between sensory inputs and the hypothalamic-pituitary axis and the pulsatile release of GnRH from the hypothalamus. In the mare during the normal period of seasonal cyclicity, melatonin secretion from the pineal gland is suppressed because of the extended photoperiod, thus allowing constant pulses of GnRH to be released from the hypothalamus (Strauss et al., 1979). Release of GnRH from the hypothalamus

occurs either from the collection of neurons that make up the arcuate and ventromedial nuclei responsible for the tonic releases of GnRH or the superchiasmatic nucleus, which is referred to as the surge center (Senger, 2005). These nuclei reside in the medial basal hypothalamus. The axons of the neurosecretory cells reach into the periventricular space in the median eminence and GnRH is contained within vesicles in the median eminence (Strauss et al., 1979). Release of GnRH from these vesicles occurs in a pulsatile manner. Hypothalamic neurons communicate with the anterior pituitary through a specialized and localized blood circulation system referred to as the hypothalamo-hypophyseal portal system (Senger, 2005). Minute concentrations of GnRH are necessary to provoke an effect on gonadotroph cells. This results from the specialized circulation in close proximity between the hypothalamus and the anterior pituitary, which GnRH stimulates its effect before dilution in systemic circulation. The 5- to 10-min half-life of GnRH (Conn et al., 1987), coupled with the restriction of its action in the hypothalamo-hypophyseal portal system before going into systemic circulation, makes it virtually undetectable in the peripheral blood supply (Ginther, 1992). The stage of the mare's estrous cycle influences from which hypothalamic center GnRH is release and at what frequency. Anestrous mares secrete GnRH from the tonic centers in a low amplitude pulsatile manner with long periods of time between releases. Anestrous mares may experience a single release every 8 h that will increase to four pulses per 8 h as mares transition to normal estrous activity (Fitzgerald et al., 1983). In normal, cycling mares, GnRH is secreted continuously with additional pulses occurring as infrequently as once daily in diestrous mares (Alexander et al., 1990). Pulsatile frequency increases to once every 1 to 2 h in mares in the peri-ovulatory period, and occurs as frequently as twice per hour in mares at ovulation (Ginther, 1992). When threshold circulating levels of estrogen (E) are reached in the absence of progesterone (P4), the increased pulse frequency of

GnRH stimulates a similar increased pulse frequency of LH (luteinizing hormone). The LH pulses become too frequent for complete removal of LH from circulation prior to the next release and the LH concentration builds up to threshold levels. Once threshold LH levels are reached, the cascade of events leading up to ovulation is initiated. Increased GnRH stimulates the production and secretion of gonadotropins from the anterior pituitary and has a controlling influence over these gonadotropin hormones. Actions of these gonadotropins subsequently control other reproductive hormones (Senger, 2005). The classical form of GnRH1 is considered the major hormone regulator of reproduction; however, recent research has identified a second isoform, GnRH2 and its associated receptor, that is not involved in gonadotropin stimulation in the pituitary (Desaulniers et al., 2017).

Gonadotropins

Primary gonadotropins produced by gonadotroph cells in the anterior lobe of the pituitary are follicle stimulating hormone (FSH) and LH. These gonadotropins are primarily responsible for folliculogenesis and steroid production (Senger, 2005). Both LH and FSH are glycoproteins consisting of two subunits. The alpha subunit of both of these hormones is conserved (Stewart et al., 1987), whereas differing beta subunits give each glycoprotein high specificity and function (Sherwood and McShan, 1977). Secretion of GnRH from the hypothalamus through the hypothalamo-hypophyseal portal system causes upregulation of both FSH and LH production and secretion in the anterior pituitary. Both FSH and LH are similarly upregulated by GnRH but are also controlled by other hormones, such as inhibin and E, that differentially influence either FSH or LH production or activity (Ginther, 1992). During the estrous cycle, FSH is generally regarded to be released in a bi-modal wave pattern (Burns and Douglas, 1981) with basal and

pulsatile secretion are controlled by dual mechanisms (Padmanabhan et al., 1997). Pulsatile releases of LH are attributed to pulsatile releases of GnRH (Clayton and Royston, 1989).

The target tissue for FSH is the granulosa cell in the antrum of a follicle (Senger, 2005). Binding of FSH occurs on plasma membrane bound receptors located on the granulosa cell membranes (Ulloa-Aguirre et al., 2013). Increased FSH concentrations stimulate growth and recruitment of a cohort of immature follicles and maturation of the primordial germ cell. As FSH rises and stimulates follicular growth, an associated rise in inhibin from the dominant follicle downregulates the secretion of FSH at the level of the anterior pituitary (Donadeu and Pedersen, 2008). Only more mature follicles can still respond to the decreasing concentrations of FSH, so only the most mature follicle(s) is selected to become dominant, with the more immature follicles no longer able to be rescued by declining concentrations of FSH and therefore undergo atresia. If the dominant follicle in the preovulatory wave is ablated, other smaller follicles may go on to become dominant and ovulate, demonstrating that all follicles have the ability to reach dominance (Ginther et al., 2003).

The target for LH is the plasma membrane receptor located on the theca interna cells of the antral follicle and the luteal cells of the corpus luteum (Senger, 2005). Release of LH occurs in a pulsatile fashion synchronous with GnRH pulse secretions (Clayton and Royston, 1989) with a half-life lasting approximately 1 h (Cole and Hart, 1930). When LH concentrations are elevated before ovulation, pulse frequency is too rapid to detect in systemic circulation (Fitzegerald et al., 1983). Concentrations of LH show a long, slow rise, peaking before ovulation (Irvine and Alexander, 1994) and reaching maximum concentrations 1 to 2 d post-ovulation followed by a rapid decline during diestrus, which is defined as the period of sustained luteal function and maximum P4 production (Palmer, 1978). Circulating concentrations of LH during anestrus are

similar to concentrations during diestrus (Garcia and Ginther, 1976). The LH surge initiates the ovulatory cascade and subsequent formation of the corpus luteum (CL) and P4 production by the CL. Circulating LH also works in concert with FSH for final follicular maturation and ovulation (Senger, 2005).

Steroid Hormones

Steroid hormones are synthesized from cholesterol by a series of complex enzymatic processes (Senger, 2005). Steroid hormones produced in the mare include testosterone (T), estrogens, and progestins (Ginther, 1992). Estradiol-17β (E2) is the estrogen of interest in the estrous mare and is produced from the enzymatic conversion of testosterone by the granulosa cells of an antral follicle under the influence of FSH and LH (Short, 1961). Target tissues of E2 include the hypothalamus, where it has a positive feedback effect on the release of GnRH and enhances the response of LH to GnRH, and also tissue throughout the reproductive tract of the mare, where it increases secretory activity and enhances uterine motility (Thompson et al., 1991). Circulating E2 causes decreased uterine tone and relaxation of the cervix and is also responsible for inducing sexual behavior and receptivity to mating (Senger, 2005). The day before follicular deviation, E2 concentrations begin to rise and reach peak concentrations before ovulation (Ginther et al., 2001). Estrogen concentrations rise concurrently with LH (Noden et al., 1978). Increased FSH also contributes to increased production of E2 at the level of the dominant follicle (Senger, 2005).

Progesterone is the progestin of interest in the non-pregnant mare and is the primary steroid hormone during diestrus. Following ovulation, granulosa and theca cells within the evacuated follicle luteinize and begin secreting P4. Production of P4 increases during the first 24 to 36 h post-ovulation (Short, 1961), reaching peak values of 4-22 ng/mL by 5 to 7 d post-

ovulation (Ginther, 2009). Target tissue for P4 includes the uterus and cervix of the reproductive tract and neuropeptides at the level of the hypothalamus. Circulating P4 is responsible for increased uterine and cervical tone in the diestrous mare (Ginther, 1992). In addition, P4, in concert with decreased E2, suppresses behavioral estrus and receptivity to a stallion. In addition, P4 serves as a strong negative feedback on GnRH release from the hypothalamus; therefore, also exerting a strong negative feedback on LH. Concentrations of P4 rise as LH decreases and fall as LH increases (Noden et al., 1978).

Additional Hormones of Reproductive Interest

Other hormones of reproductive interest that are neither categorized as gonadotropins or steroid hormones are involved in regulation of the estrous cycle in the mare. Inhibin is a glycoprotein produced by the granulosa cells in antral follicles on the ovary. As a dominant follicle approaches ovulation, increased synthesis of inhibin by these cells occurs (Ginther, 1992). The target of inhibin is the anterior lobe of the pituitary where it inhibits FSH secretion but has a minimal effect on LH (DeJong, 1987). Circulating concentrations of inhibin are inversely related to circulating FSH and the period of greatest circulating inhibin occurs at ovulation, rapidly declining post-ovulation (Bergfelt et al., 1991).

Prostaglandins are lipids that consist of 20-carbon fatty acids and are derived from arachidonic acid (Pike, 1971). Many different types of prostaglandins are involved in a wide array of physiological processes (Senger, 2005). Two prostaglandins of particular reproductive interest are prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and prostaglandin E_2 (PGE_2). These prostaglandins are produced by the ovary and uterine endometrium in the non-pregnant mare. The mode of action of prostaglandins is generally via an autocrine or paracrine function (Ginther, 1992). One target for $PGF_{2\alpha}$ is the CL where it initiates luteolysis at approximately d 14 of the estrous cycle if the

mare is not pregnant (Ginther, 2009). Increased $PGF_{2\alpha}$ also promotes uterine contractions and increases contractions of ovarian smooth muscle to increase follicular pressure before ovulation. With regard to its role in ovulation, $PGE_{2,}$ in addition to histamines produced by the ovary and uterus, stimulate increased localized blood flow to the ovary including the dominant follicle, thereby increasing both edema and intra-follicular pressure before ovulation. Circulating PGE_2 is also involved in remodeling of the evacuated follicle to form the CL and stimulating secretion of P4 by the CL by inducing LH receptors in the CL, leading to LH-induced P4 production (Niringiyumukiza et al., 2018).

Estrous Cycle of the Mare

Mares are seasonally polyestrous, which is characterized by regular estrous cycles during late spring, summer, and early fall followed by cycle quiescence during winter months. Quiescence is bracketed by both a spring or vernal transition and a fall transition period. During the fall transition, mares generally experience erratic estrous cycles and finally cease cycling and transition to anestrus during late fall and winter months (King, 2011). Following a period of winter anestrus, mares will undergo another transition in the spring months. The increase in day length and exposure to increasing amounts of light stimulates the pineal gland of the mare resulting in a reduced melatonin production. External light stimulus is transmitted from the superchiasmatic nucleus to the paraventricular nucleus (PVN), then from the PVN to the superior cervical ganglia and finally to the pineal gland. Melatonin production in the pineal cells decreases in long-day breeders in response to increased daily light duration (Clifton and Steiner, 2009). Reduced melatonin decreases its suppressive effect on GnRH secretion and release from the hypothalamus. Release of increased amounts of GnRH stimulates both FSH and LH production, which are responsible for recruiting and selecting a dominant follicle to grow and

ovulate for the first ovulation of the season. After transitioning to normal cyclic activity, a mare will continue to cycle every 21 d (± 3 d) until she enters the fall transition followed by seasonal anestrus during late fall and winter months (Ginther, 1992).

Follicular Phase

A typical 21-d estrous cycle is illustrated in Fig. 1.1. It is categorized by two distinct phases based on the dominant steroid hormone influencing the cycle during each phase. The follicular phase includes the period of time from CL regression to ovulation, and consists of the stages of proestrus and estrus. This phase accounts for approximately 20% of the estrous cycle and lasts 7.7 ± 2.9 d in mares (Ginther et al., 1972). During the follicular phase, P4 declines rapidly during luteolysis, which upregulates gonadotropin release from the hypothalamus, followed by an increase in FSH and LH release from the anterior pituitary to stimulate follicular growth and maturation of the preovulatory follicle in preparation for ovulation (Alexander and Irvine, 2011). Increased production of E2 begins 6 to 8 d before ovulation, reaching peak concentrations 2 d before ovulation (Makawiti et al., 1983). Increased E2 during the follicular phase, in the absence of negative inhibition from P4, stimulates sexual behavior and receptivity, and has a negative feedback effect on FSH release (Senger, 2005). Release of FSH is also negatively impacted by increased inhibin production from the dominant follicle. Increased E2 concentrations also have a positive feedback effect on GnRH and LH, initiating the preovulatory surge of these hormones, which initiates the ovulatory cascade. That cascade will be reviewed in a subsequent section.

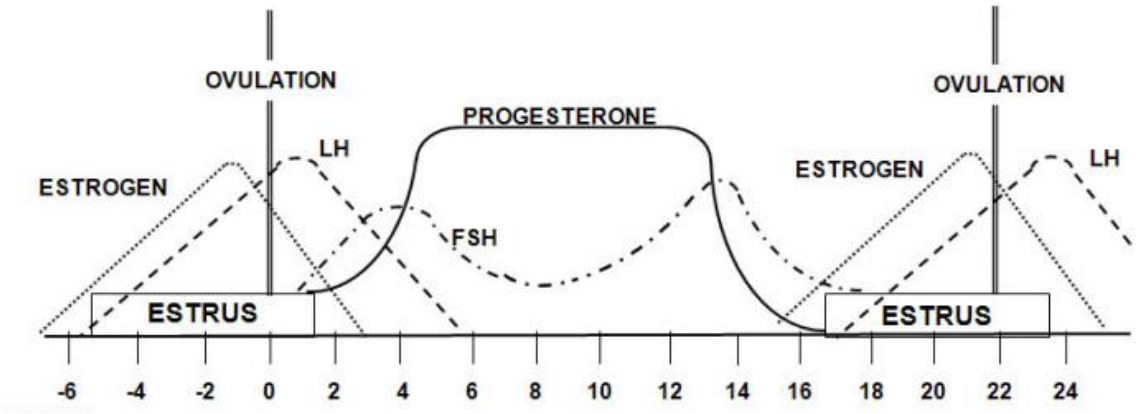

Figure 1.1 Circulating hormones during the estrous cycle of the mare – Adapted from Taylor-MacAllister and Freeman.

Luteal Phase

Post-ovulation, the estrous cycle shifts from the follicular phase to the luteal phase which consists of the stages of metestrus and diestrus. The luteal phase lasts 15.8 ± 2.5 d and accounts for approximately 80% of the duration of the estrous cycle (Ginther et al., 1972). Metestrus is characterized by CL formation because of luteinization of the granulosa and theca cells from the now evacuated dominant follicle and the switch from E2 to P4 dominance, with increasing amounts of P4 now being produced by the CL. Progesterone secretion rises rapidly following ovulation, reaching peak concentrations by 8 d post-ovulation (Lofstedt, 2011). Diestrus is characterized by sustained luteal P4 production and maximal luteal function and generally lasts 15 ± 3 d (Loftstedt, 2011). The release of $PGF_{2\alpha}$ from the uterine endometrium at approximately d 14 after ovulation is responsible for luteolysis. Luteolysis causes a drastic and immediate drop in circulating P4, removing the negative feedback for GnRH secretion and the inhibition of

behavioral estrus. Luteolysis terminates diestrus and the cycle is shifted back to the follicular phase as a new dominant follicle is matured and prepared for ovulation (Senger, 2005).

Follicular Development in the Mare

Folliculogenesis can be described as the process by which immature follicles develop into more advanced follicles and are either selected to become candidates for ovulation or undergo atresia (Senger, 2005). Follicular waves may be classified as major or minor, with major waves being the only ones that can produce dominant follicles that go on to ovulate (Donadeu and Pedersen, 2008). For this review, the focus will be on the major follicular wave. There are several major periods that occur during folliculogenesis and they can be defined as recruitment, emergence, selection, deviation and dominance (Evans, 2003; Donadeu and Pedersen, 2008). Some researchers characterize recruitment and emergence as a single phase (Ginther et al., 1996) but others have defined recruitment as a separate phase defined by the growth of follicles that have become dependent on gonadotropin support (Driancourt, 2001). Emergence is characterized as the day when a cohort of follicles develops an antrum and are able to be visualized using ultrasonography (Evans, 2003). Generally, emergence in the horse is defined as the time period when a follicle reaches 6 mm (Ginther et al., 2003). Once the cohort of follicles emerges, common growth of these follicles is observed at a rate of 2 to 3 mm per day (Donadeu and Pedersen, 2008) for approximately 6 d (Ginther, 2000). Reduction in the number of follicles in the cohort is described as selection of the dominant follicle. At the end of selection, deviation of diameter of the largest and second largest follicle is observed. Deviation is generally characterized as beginning 7 d before ovulation, when a follicle reaches 22 mm in diameter, or both (Ginther, 2003). Growth of the selected follicle remains constant and the subordinate follicles arrest and undergo atresia. The single follicle now achieves dominance and will grow to

approximately 35 to 45 mm before ovulation under the influence of E2 and LH (Donadeu and Pedersen, 2008).

Ovulation in the Mare

Unique to the mare, ovulation is restricted to the ovulatory fossa limiting them to ovulation of a single dominant follicle from the ovary at a given time. Double ovulation, or the ovulation of a follicle from each ovary, either synchronously or asynchronously, is not uncommon, but would not be classified as a normal occurrence. Although not common, a mare also may experience asynchronous ovulations of multiple follicles from the same ovary. A primary double ovulation is generally characterized as ovulations that are 1 to 2 d apart (Ginther, 1992). Rate of double ovulations may be affected by several factors, such as breed and previous history of multiple ovulations, and has been reported to range from 8 to 25% (Ginther, 1986).

Ovulation is characterized as a cascade of events including a complex regulatory system under hormonal control. As previously described, threshold concentrations of E2 produced by the dominant follicle positively influence the surge center of the hypothalamus, initiating the release of large quantities of GnRH in a preovulatory surge. The surge of GnRH initiates a surge release of LH (at greater than 10 times basal concentrations) from the anterior pituitary. Dominant follicles also produce angiogenic factors and histamines that promote generation of new blood vessels surrounding the follicle to increase localized blood flow (Senger, 2005). Increased PGE_2, upregulated by the preovulatory surge of LH, stimulates hyperemia to the ovary and in particular the dominant follicle. Hyperemia from angiogenesis and vasodilation provide the dominant follicle with the hormonal stimulus and metabolites necessary for final maturation and ovulation. Circulating PGE_2 also promotes increased edema that contributes to increased intrafollicular pressure eventually involved in antrum rupture. Another prostaglandin, $PGF_{2\alpha}$ is

synthesized and released locally by the ovary to stimulate contractions of ovarian smooth muscle that also contributes to increased intrafollicular pressure. Circulating $PGF_{2\alpha}$ also stimulates release of lysosomal enzymes from granulosa cells that are responsible for connective tissue degradation, thereby weakening the follicular wall (Murdoch and Gottsch, 2003). Within the dominant follicle, the shift from E2 production by granulosa cells to small amounts of P4 from theca interna cells upregulates the production and release of collagenases that break down collagen and further weakens cell membranes. Increased follicular pressure and follicular wall weakening occur. Visualization of the pre-ovulatory follicle by ultrasound imaging generally shows a softening of the follicle and irregularity in shape (Ginther, 1986). Breakdown of the follicular wall eventually leads to follicular rupture and collapse which characterizes ovulation. The oocyte is released from the follicle through the ovulatory fossa to enter the oviduct (Senger, 2005).

Once ovulation has occurred, a corpus hemorrhagicum (CH) forms briefly at the site of the evacuated follicle. The CH develops because of localized hemorrhaging from small blood vessels rupturing during and following ovulation. The same steroidogenic cells in the dominant follicle that are involved in the production of T and E2, and a small amount of P4, are also responsible for steroidogenesis of P4 during the luteal phase. Circulating LH stimulates luteinization of the evacuated follicle with theca interna cells transitioning to small luteal cells and granulosa cells transitioning to large luteal cells. Both of these cell types are responsible for P4 production, which will increase rapidly over the next several days and reach peak concentrations 5 to 6 d post-ovulation (Senger, 2005).

Ovulation Synchronization in the Mare

Ovulation synchronization in the mare is a useful technique for several applications. Ovulation control is useful in a timed breeding scenario, including breeding with frozen semen or dealing with limited stallion availability resulting from events such as competition schedules. Ovulation synchronization is also necessary and utilized extensively when synchronizing a donor mare and recipients for use in an embryo transfer program. Synchronization of ovulation in the mare can be more difficult than in other species because of prolonged estrous and diestrous periods (Ginther, 1992). In addition, some mares also are able to ovulate during diestrus (Claes et al., 2017), decreasing the efficacy of a protocol. Insemination of the mare is the most successful when conducted during a short period of time close to ovulation. The best chance of conception occurs when a mare is inseminated from 24 before up to 12 h post-ovulation (Sieme et al., 2003); however, clinical recommendations are generally to inseminate 48 h prior and up to 6 h post-ovulation.

The most widely accepted and used protocol for synchronization in the mare is administration of exogenous progesterone and estradiol (P+E; Mottershead, 2004, Loy et al., 1981). Briefly, the 19-day protocol involves injections of 150 mg progesterone and 10 mg estradiol prepared in oil and administered daily for 10 days. A single injection of $PGF_{2\alpha}$ is administered on day 10 of P+E treatment. Mares are administered an ovulatory agent on d 18 and inseminated on d 19. The 10-d regimen of exogenous hormone administration has a negative effect on circulating gonadotropins and suppresses follicular activity (McCue, 2014). Approximately 85% of mares will respond appropriately to this synchronization protocol. In addition to a possible failure to respond to synchronization, this protocol requires daily handling

of horses and daily administration of intramuscular drugs. These pharmacological agents can also cause injection site reactions, which may become infected and painful (McCue, 2014).

Other synchronization protocols commonly used are a single injection of $PGF_{2\alpha}$, commonly referred to as short cycling a mare, or two $PGF_{2\alpha}$ injections spaced 12 d apart. Administration of $PGF_{2\alpha}$ may have negative side effects when administered to some horses, such as sweating, abdominal discomfort, and diarrhea (McCue, 2014). Effectiveness of $PGF_{2\alpha}$ treatment depends on stage of the estrous cycle at the time of administration and requires a functional CL. Luteolysis initiated by exogenous $PGF_{2\alpha}$ administration will only be effective from approximately d 5 when the CL acquires functional receptors for $PGF_{2\alpha}$ to d 14 of the estrous cycle, when endogenous $PGF_{2\alpha}$ release will initiate luteolysis. In addition, variability exists in the return to estrus depending on follicle size/maturation level at the time of $PGF_{2\alpha}$ administration (Loy et al., 1981).

A final common synchronization protocol calls for the administration of P4 via an oral or injectable route to maintain elevated P4 concentrations for 14 d. Administration of $PGF_{2\alpha}$ is concurrent with the final day of P4 treatment. In some mares, P4 alone isn't able to suppress ovulation and they may ovulate during treatment (Hughes et al., 1972). On the other hand, a mare in late estrus may respond to P4 administration by holding a large, pre-ovulatory follicle for much longer than normal and when stimulated to ovulate, ovulate an aged oocyte that may lead to decreased embryo survival if the oocyte is fertilized (Carnevale, 2008). Long-term administration of P4 may also lengthen the interval to ovulation by long-term suppression of circulating LH (McCue, 2014). This protocol also requires daily handling and administration of drugs if the P4 is administered orally. Injectable forms of P4 limit the amount of handling to one or two injections; however, injection site reactions may be observed. In addition, success of

exogenous P4 administration to synchronize ovulation is only effective during certain times of the year when a normal cyclic activity has been established.

Transvaginal Ultrasound-Guided Follicular Ablation

Protocol

Follicular ablation (FA) has been used recently as a synchronization tool in other species and has the advantages of reduced animal handling requirements, little to no use of exogenous hormones and no dependence of the animals' estrous cycle stage (Bergfelt et al., 2007). While the specific equipment may vary, the procedure generally follows a standard protocol. For this procedure, a mare is restrained in stocks and sedated, generally with the addition of an antispasmodic agent, and the perineum is washed. Usually an analgesic such as lidocaine is applied to the vaginal wall before the introduction of the transducer. The ultrasound transducer is then inserted vaginally while the ovary is manually manipulated adjacent to the vaginal wall by rectal palpation. A needle is advanced through the transducer, and guided by the ultrasound image of the ovary, through the vaginal wall and into the ovary. Care is taken to pass through as much ovarian stroma as possible before puncture of the follicle, particularly in procedures where follicle rupture or total ablation is not the objective. Based on the aim of the procedure, follicular contents such as oocytes and follicular fluid may now be aspirated with the use of a vacuum pump or simple backpressure applied with a large syringe, or medications and/or oocytes may be placed into the penetrated follicle. There are many factors that can and do affect the success of the procedure. These can include equipment choices such as needle size, treatment differences such as hormone administration and aspiration frequency, and individual animal variation such as breed, stages of the estrous cycle and follicular size (Squires and Cook, 1996).

While rare, there are a few complications that have been noted with this procedure. Because a follicle, particularly a periovulatory follicle, has a highly vascularized follicular wall, small amounts of blood may be appreciated in collected follicular fluid. The presence of large amounts of blood could indicate more severe problems such as internal hemorrhaging (Vanderwall and Woods, 2002). Other complications that have been reported include ileus, peritonitis and colic (Carnevale, 2008). No significant effects on fertility in the mare have been found on subsequent inseminations following FA (Mari et al., 2005, Vanderwall et al., 2006).

Application of Follicular Ablation

With the addition of FA as an assisted reproductive technique, the procedure has been used in a number of species both clinically and experimentally, to investigate a variety of objectives (Carnevale, 2008). In cattle, FA has been used to study gamete recovery and intrafollicular oocyte transfer in heifers (Bergfelt et al., 1998), follicular fluid hormone dynamics (Aller et al., 2013), as a technique for intrafollicular drug administration (Kot et al., 1995) and to study luteal function (Bisinotto et al., 2012). Additional studies in cattle have shown the use of FA before superovulation increased the number of ova/embryo output, albeit mostly nontransferable ova (Shaw and Good, 2000). The FA procedure also improved ovarian response as evidenced by increased production of oocytes and increased the number of embryos collected in early lactation (Amiridis et al., 2006). Ablation also has been reported to be as efficacious as P+E in synchronizing follicular wave emergence in cattle (Baracaldo et al., 2000), and it improved the superovulatory response in cattle as evidenced by a greater number of ova and embryos collected (Sendag et al., 2008, Lima et al., 2007). In pigs, FA has been utilized to study its effects on post-weaning anestrous intervals (Cox et al., 1987). Ablation has also been used in

estrous synchronization in wapiti (McCorkell et al., 2008) and to collect oocytes from superstimulated bison (Palomino et al., 2016).

The FA procedure also has extensive application for clinical and experimental purposes in the horse. Research benefits include studying follicular dynamics (Ginther et al., 2009) and injection of pharmacological agents directly into follicles (Ginther et al., 2014). Unlike other species, horses respond poorly to superovulation protocols and are bound by the anatomical and physiological limitations of the ovulatory fossa, making the collection of multiple embryos at a single time point difficult. Equine oocytes and embryos also have poor maturation rates *in vitro* (Deleuze et al., 2009). These two reproductive hurdles have been targeted for study using the FA procedure in combination with other *in vivo* techniques such as intra-oviductal oocyte transfer and intra-follicular oocyte transfer to circumvent inadequacy *of in vitro* fertilization in horses (Deleuze et al., 2009, Carnevale et al., 2000, Hinrichs and DiGiorgio, 1991). Due to an extended period of seasonal anestrus, FA provides the opportunity to collect oocytes from mares that are not cycling and also while carrying a pregnancy (Purcell et al., 2007).

Follicular ablation has been used in several species to initiate a new follicular wave and synchronize a cohort of animals. Ovulation synchronization facilitated by FA has been shown to be successful in cattle (Martinez et al., 2000, Bergfelt et al., 1994). Ablation has been more widely used in cattle to initiate a new follicular wave for superovulation treatment in an embryo collection program (Barcaldo et al., 2000, Bergfelt et al., 1997). The initial follicular wave in cattle is less variable, and when initiation of the first follicular wave is synchronized, the total group synchrony is tighter. Variability in synchronization protocols can potentially be attributed to stage of the estrous cycle and corresponding follicular development at the onset of drug administration.

In a previous report, use of FA was applied to synchronize a large group of mares on random days of the estrous cycle (Bergfelt et al., 2007). Following the ablation procedure, the FSH surge and deviation of the future dominant follicle were more synchronized, LH concentrations were greater before and after deviation, E2 concentrations were greater following deviation and follicles grew at a faster rate (Ginther et al., 2008, Cook 1995, Hinrichs et al., 1991). Removal of the dominant follicle(s) is not only useful for synchronizing ovulation in mares but can also have potential application for delaying ovulation. By utilizing FA to remove the dominant follicle(s) destined to ovulate, the estrous cycle is "restarted" and time to ovulation could be longer than letting the dominant follicle continue to ovulation.

Use of Follicular Ablation in the Postpartum Mare

In many disciplines, such as racing and showing, there are competitive advantages related to size and training level for foals born earlier in the year compared with their later counterparts, especially when sold at yearling sales or when raced or competed as 2 and 3 yr olds. Therefore, a need exists to have a mare conceive and foal as close to January 1 as possible. For a mare to maintain an early annual foaling interval, she must become pregnant again within weeks of parturition. Most mares have normal follicular development and ovulate during this initial postpartum interval, referred to as foal heat, by day 20 postpartum (Loy, 1980). Timing of the first postpartum ovulation has a significant effect on pregnancy rates associated with that ovulation. If a mare ovulates before day 5-6 or 7-8 postpartum, there is a 0% and 32% chance of conception, respectively. If ovulation occurs 9-12 days postpartum, conception rates rise to 60%, and when ovulation occurs later than day 12, conception rates rise again to 75%, which is considered an acceptable rate and would be comparable with other normal ovulations (Zent, 2006). The lower pregnancy rates associated with an early ovulation are due to a number of

factors including incomplete uterine involution, fluid sequestered in the uterine lumen, infection, inflammation and other traumatic events, such as cervical and uterine trauma, around foaling (Zent, 2006). Mares have the best chance of conceiving on foal heat and maintaining a yearly foaling interval if the interval from foaling to ovulation is at least 12 days but less than 25 days. If the initial postpartum ovulation could be delayed, allowing for a greater chance of conception without the added time associated with electing to breed on the mare's second natural cycle, foaling the subsequent year could take place within the appropriate time period.

Several hormone administration protocols exist to lengthen the interval from parturition to ovulation in the mare. Exogenous oral or injectable P4 and P+E protocols, as previously described, have successfully lengthened this interval; however, administration of exogenous progesterone may close a mare's cervix at a time period when an open cervix is necessary for uterine clearance of debris from the foaling process, potentially creating uterine infections and other issues that would negatively impact future fertility (Zent, 2006).

Follicular ablation has been performed successfully in several species such as cattle (Martinez et al 2000, Barcaldo et al., 2000, Bergfelt et al., 1994) and horses (Carnevale, 2008, Bergfelt et al., 2007). This procedure has been used to synchronize ovulation, initiate new follicular waves for superovulation treatment, and for oocyte collection and transfer. Further investigation into the comparison of FA to conventional P+E protocols for ovulation synchronization is warranted. Studies have not yet been conducted to determine if follicular ablation could be useful in delaying the postpartum interval to ovulation in the mare.

Chapter 2 - Application of the follicular ablation technique in mares

Abstract

Two experiments were conducted to determine the practicality of utilizing transrectal ultrasound-guided follicular ablation in a commercial setting. The objective of the initial experiment was to investigate the efficacy of follicular ablation as a technique for ovulation synchronization when compared with a standard progesterone and estrogen (P+E) protocol. Twenty non-pregnant mares were assigned to an ovarian follicular ablation (FA, n = 10) or P+E treatment (n = 10). Briefly, FA mares were subjected to follicular ablation to remove all follicles > 10 mm on d 1 and administered prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) twice on d 5. Mares were administered 2,500 IU human chorionic gonadotropin (hCG) on d 11 and evaluated via ultrasound twice daily until ovulation was detected. Mares in the P+E treatment were scanned at initiation of the protocol and received P+E IM once daily for 10 d. On d 10, mares received $PGF_{2\alpha}$ and ultrasound monitoring began on d 15. On d 18, mares were administered hCG and evaluated twice daily until ovulation was detected. Interval from initiation of treatment to ovulation (10.4 vs 19.1 d) and the interval from hCG administration to ovulation (28.8 vs. 55.5 h) was shorter ($P<0.01$) in the FA mares versus the P+E mares, respectively. Ablation may be an acceptable non-steroidal alternative to the conventional P+E protocol and can shorten the interval from treatment to ovulation.

The objective of the second experiment was to determine if ablation could prolong the interval from parturition (d 0) to the first postpartum ovulation. Eighteen postpartum mares were assigned to a FA treatment (n = 10) or untreated control (CON; n = 8). On d 6, FA mares were subjected to follicular ablation to remove all follicles > 10 mm. Mares were administered $PGF_{2\alpha}$ twice on d 11 and monitored via ultrasound once daily until a follicle ≥ 35 mm was detected, at

which time mares were treated with 2,500 IU of hCG. After hCG, mares were monitored twice daily until ovulation was detected. The CON mares were evaluated using ultrasound beginning on d 4, continuing every other day until a follicle ≥ 30 mm was detected at which time scanning frequency increased to once daily. When a follicle ≥ 35 mm was identified, mares were administered hCG and monitored twice daily until ovulation was detected. Interval from foaling to ovulation was longer (P<0.01) in the FA mares (15.9 d) compared with CON mares (10.0 d). Increasing the interval from foaling to ovulation is known to increase conception rates; therefore, application of this procedure could be utilized to optimize the timing of breeding, thereby improving overall pregnancy rates. Results from these experiments support the commercial application of follicular ablation in synchronization programs and delaying ovulation in the early postpartum mare.

Keywords: mare, follicle ablation, postpartum ovulation, synchronization

Introduction

Transvaginal ultrasound-guided follicular ablation (FA) is a relatively low risk procedure that has been studied in many species and has been used for a variety of objectives. Initially developed as a research tool, FA was first studied as a method to collect embryos from cattle (Pieterse et al., 1988). In the horse, it has been used for a number of clinical and experimental purposes. Research applications include studying follicular dynamics (Ginther et al., 2009a,b) and intra-follicular administration of pharmacological agents (Ginther et al., 2014), and oocyte collection and intra-follicular transfer (Carnevale, 2004). Compared with other species, horses respond poorly to superovulation protocols and are bound by physiological limitations of the ovulatory fossa, making collection of multiple embryos at a single time point difficult (Ginther, 1992). Equine oocytes and embryos also have poor maturation rates *in vitro* (Deleuze et al.,

2009). Using the FA procedure in combination with other *in vivo* techniques, such as intra-oviductal oocyte transfer and intra-follicular oocyte transfer, researchers have attempted to overcome obstacles associated with *in vitro* oocyte fertilization in mares (Hinrichs and DiGiorgio, 1991, Carnevale et al., 2000, Deleuze et al., 2009). Because mares are also seasonally polyestrous, the FA technique offers the ability to collect oocytes from mares during anestrus (Purcell et al., 2006). Using the FA procedure, collection of oocytes from mares carrying a pregnancy also has been reported (Purcell et al., 2006). Collected oocytes may be frozen, fertilized *in vitro*, or utilized for intra-follicular oocyte transfer.

Traditional synchronization protocols in mares and other species involve the repeated administration of exogenous hormones. The most widely accepted protocol for synchronization of ovulation in the mare is administration of exogenous progesterone and estradiol (P+E; Mottershead, 2004). Briefly, the 19-d protocol involves injections of 150 mg progesterone and 10 mg estradiol prepared in oil and administered daily for 10 d. A single injection of $PGF_{2\alpha}$ is administered on day 10 of P+E treatment. Mares are administered an ovulatory agent on d 18 and inseminated on d 19. The FA procedure offers an alternative method for initiating a new follicular wave (Bergfelt et al., 1997), and also removes the necessity of exogenous steroid administration and decreases the labor associated with daily handling. Following ablation in the mare, the FSH surge and deviation of the dominant follicle were more synchronized, LH concentrations were greater before and after deviation, E2 concentrations were greater following deviation and follicles grew at a faster rate (Hinrichs et al., 1991, Cook 1995 Ginther et al., 2008). A previous study investigated the use of FA to synchronize a large group of mares and acceptable synchronization rates were achieved (Bergfelt et al., 2007). The objective of

experiment 1 was to determine if FA achieved similar or enhanced ovulation synchrony compared with the current P+E treatment.

In many disciplines, such as racing and competitive showing, earlier born foals have the advantage of both size and level of training over their counterparts born later in the year. For foals to be born early in the year, mares must conceive by early February to foal as close as possible to January 1 of the following year. For a mare to maintain a yearly foaling interval, on average, she must become pregnant again within 25 d of parturition. Most mares have normal follicular development and ovulate by d 20 postpartum (Loy, 1980). Timing of first postpartum ovulation has a significant effect on fertility associated with that ovulation. If ovulation occurs beyond d 12 postpartum, pregnancy rates increase to more acceptable values (Loy, 1980). The poorer pregnancy rates associated with earlier ovulation result from a number of factors including incomplete uterine involution, fluid sequestered in the uterine lumen, infection, inflammation and other traumatic events, such as cervical trauma and tearing, around the time of foaling (Vanderplassche et al., 1983, Zent, 2006).

In most cases, by extending the interval from parturition to ovulation by only a few days, normal pregnancy rates can be achieved, eliminating the need to postpone breeding until the second postpartum estrous cycle. Several hormone protocols exist that can lengthen the interval from parturition to ovulation in the mare. Exogenous P4 and P+E protocols successfully lengthen this interval; however, administration of exogenous P4, particularly to mares with a history of poor uterine clearance, may increase the tone of a mare's cervix and decrease uterine motility, negatively impacting uterine clearance (McKinnon et al., 1988), and poorer immune function, reducing the ability of the mare to clear accumulated bacteria (Leblanc et al., 2011). In addition, some mares may ovulate despite postpartum exogenous P4 administration (Loy et al., 1975).

Follicular ablation has been performed successfully in several species, including cattle (Bergfelt et al., 1994, Barcaldo et al., 2000, Martinez et al., 2000) and horses (Bergfelt et al., 2007, Carnevale, 2008). To date, no studies have been conducted to determine if follicular ablation could delay the timing of ovulation in the mare. The objective of experiment 2 was to determine if follicular ablation could delay the initial postpartum ovulation thereby increasing the chances of establishing an earlier successful pregnancy and while maintaining a yearly foaling interval.

Materials and Methods

Experiment 1

Animals and Treatments

This study was reviewed and approved by the Kansas State University Animal Care and Use Committee before study initiation. Twenty non-pregnant, stock-type mares, ranging from 8 to 20 yr old, were used for this experiment. All mares were group-housed in small lots (1 to 3 acres) with minimal grass cover and received a concentrate and forage diet formulated to meet or exceed NRC requirements (NRC, 2007). Mares had *ad libitum* access to water and a trace mineral supplement. Before initiation of the study, mares were monitored via transrectal ultrasound (Medison Sonovet 600; Samsung Medison, Ridgefield Park, NJ) equipped with a 5 MHz linear transducer until ovulation was detected. Ovulation was characterized by the disappearance of the dominant follicle and subsequent corpora lutea formation. Pre-treatment observation continued until ovulation was observed in all mares, confirming mares were cycling normally. Treatment protocols were initiated on the same day for all mares.

Following ovulation, mares were assigned randomly to the follicular ablation treatment (FA; n = 10) or to receive progesterone and estradiol treatment (P+E; n = 10). Mares in the FA treatment were scanned on d 1 of treatment and all follicles > 10 mm were targeted for removal by follicular ablation. On d 5 (4 d post-FA) each mare received two injections of $PGF_{2\alpha}$ (10 mg/dose, IM, Lutalyse®; Zoetis, Florham Park, New Jersey) 12 h apart. Six days after $PGF_{2\alpha}$ administration (d 11 post-FA), mares received hCG (2,500 IU, IV, Chorulon®; Intervet Inc., Millsboro, DE) and were monitored via ultrasound twice daily at 0800 h and 2000 h until ovulation was detected. The FA protocol (Fig. 2.1) used in this experiment was reported previously (Bergfelt et al., 2007). Mares completed the experiment once ovulation was detected.

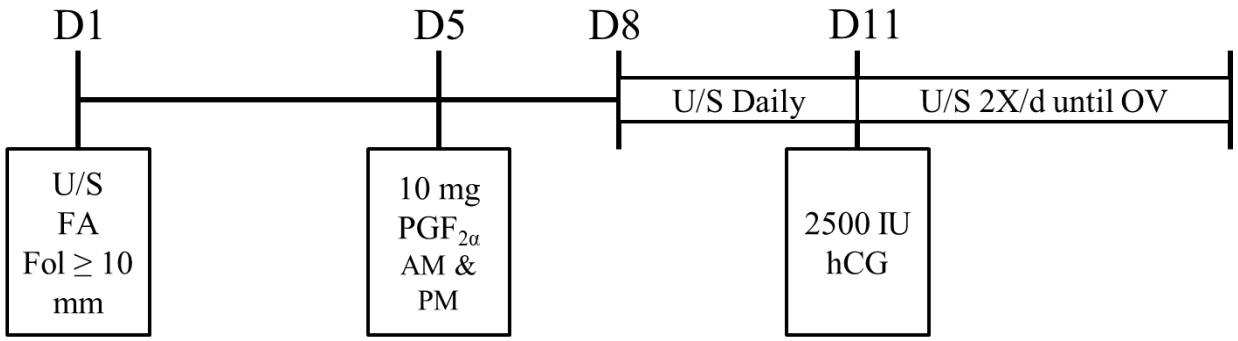

Figure 2.1 Time line for ovulation synchronization procedural design for the FA treatment

Ovaries in the P+E mares were scanned via ultrasound on the day P+E was initiated (d 1). Following a standard 19 d P+E protocol (Fig. 2.2), 150 mg of progesterone (P4) and 10 mg estradiol 17-β (E2) were administered IM at 0800 h each day for 10 d (Mottershead, 2004, Loy et al., 1981). On d 10, mares also received $PGF_{2\alpha}$ (10 mg/dose, IM, Lutalyse®; Zoetis, Florham Park, New Jersey) concurrent with the final P+E dose. Eight days after the initial prostaglandin injection (d 18), mares were administered hCG (2,500 IU, IV, Chorulon®; Intervet Inc.,

Millsboro, DE) and monitored via ultrasound twice daily at 0800 h and 2000 h until ovulation was detected. Mares completed the study once ovulation was detected.

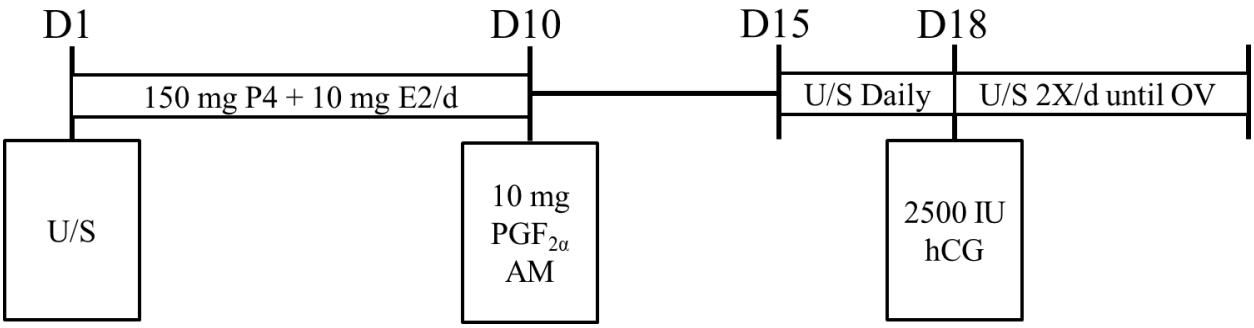

Figure 2.2 Time line for ovulation synchronization procedural design for the P+E treatment

Ablation Procedure

For the ablation procedure, mares were restrained in stocks and sedated using detomidine hydrochloride (0.01 mg/kg, IV, Dormosedan, Zoetis, Florham Park, New Jersey). In addition, N-butylscopolammonium bromide (0.3 mg/kg, IV, Buscopan, Boehringer Ingelheim, St. Joseph, MO) was administered concurrently in order to relax the rectum before manipulation of the ovary. The tail of each mare was wrapped and pulled to the side and the perineum was washed in an aseptic manner. The FA procedure was performed using an ultrasound scanner (Aloka SSD-500; Diagnostic Medical Devices, Tokyo, Japan) using a 5.0 MHz convex-array transducer equipped with a 17-gauge 53.3 cm needle. Before the transducer was introduced into the vagina, a 2% topical Lidocaine gel (Xylocaine 2% Jelly; Akorn Pharmaceuticals, Lake Forest, IL) was manually applied where the needle would puncture the vaginal wall in order to numb the area and reduce potential discomfort. Once the transducer was inserted into the vagina, the ovary selected for targeting was manipulated per rectum towards the transducer. The needle was advanced through the vaginal wall into the selected follicles (> 10 mm in diameter) and the

follicular contents were evacuated by applying back pressure with a sterile syringe connected to the tubing attached to the transducer needle.

Statistical Analyses

Data were analyzed using the GLM procedure in SAS (version 9.1, Cary, NC). Differences in treatments were evaluated using ANOVA. The model included treatment to determine the following outcomes: stage of the estrous cycle at treatment onset, interval from onset of treatment to ovulation, days from hCG administration to ovulation, hours from hCG administration to ovulation, days from initial $PGF_{2\alpha}$ administration to ovulation, diameter of follicle ovulated, incidence of double ovulation and the diameter of the follicle at hCG administration. Binomial data defined as the percentage of mares synchronized in each group at d 2 and 4 was also calculated and analyzed using Chi-square. Data are presented as least squares means ± SEM. Significance was determined at $P<0.05$.

Experiment 2

Animals and Treatments

This study was reviewed and approved by the Kansas State University Institution Animal Care and Use Committee before study initiation. For Experiment 2, 18 postpartum stock-type mares, ranging from 4 to 16 yr of age, were enrolled. All mares were group-housed in small lots with grass cover and received a concentrate and forage diet formulated to meet or exceed NRC requirements (NRC, 2007) for mares in early lactation. Mares had *ad libitum* access to water and a trace mineral supplement. Before the expected foaling date, mares were assigned randomly assigned to either of two treatments: follicular ablation (FA, n = 10) or an untreated control (CON, n = 8). All mares began the experiment at parturition (d 0).

Ovaries of FA mares were evaluated via ultrasound beginning on d 6. All follicles > 10 mm were removed on d 6 using the ablation procedure as previously described. On d 11 (5 d post-FA), each mare received $PGF_{2\alpha}$ (10 mg/dose, IM, Lutalyse®; Zoetis, Florham Park, New Jersey) at 0800 h and 2000 h. Beginning on d 12, mares were monitored via ultrasound every other day until a follicle ≥ 30 mm was first observed. Once a 30 mm follicle was detected, scanning frequency increased to once daily day until a follicle ≥ 35 mm was measured, at which time mares received hCG (2,500 IU, IV, Chorulon®; Intervet Inc, Millsboro, DE). After treatment with hCG, mares were monitored via ultrasound twice daily until ovulation was detected (Fig. 2.3).

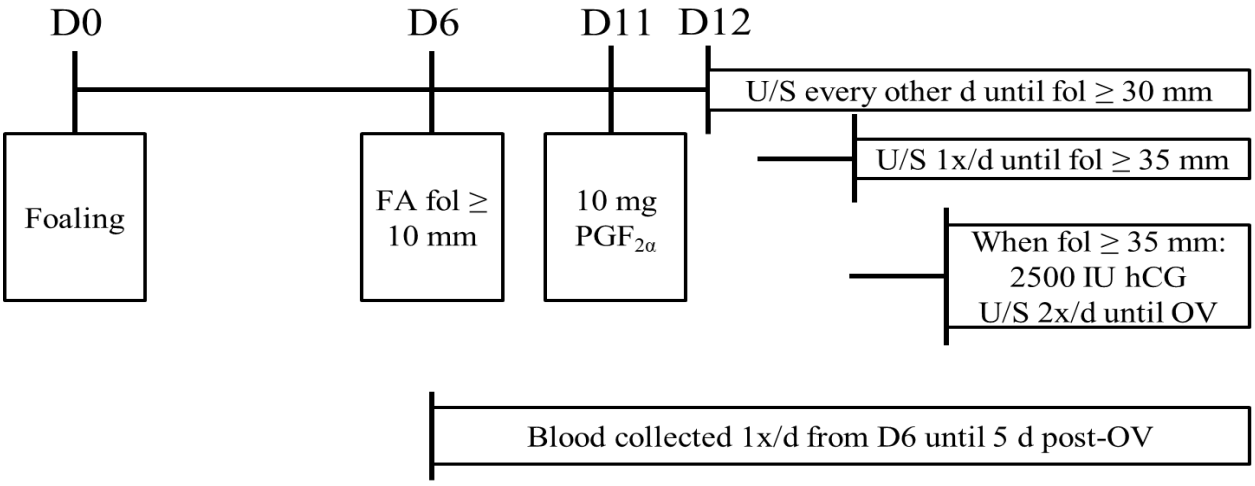

Figure 2.3 Procedural time line for delaying ovulation post-foaling using follicular ablation (FA) treatment

Control mares were scanned via ultrasound beginning on d 6. Follicular development was monitored every other day until a follicle ≥ 30 mm was detected. Once a 30 mm follicle was detected, scanning frequency increased to once daily until a follicle ≥ 35 mm was measured, at which time mares received hCG (2,500 IU, IV, Chorulon®; Intervet Inc, Millsboro, DE).

Following hCG, mares were monitored via ultrasound twice daily until ovulation was detected (Fig. 2.4). All mares completed the experiment on d 5 post-ovulation.

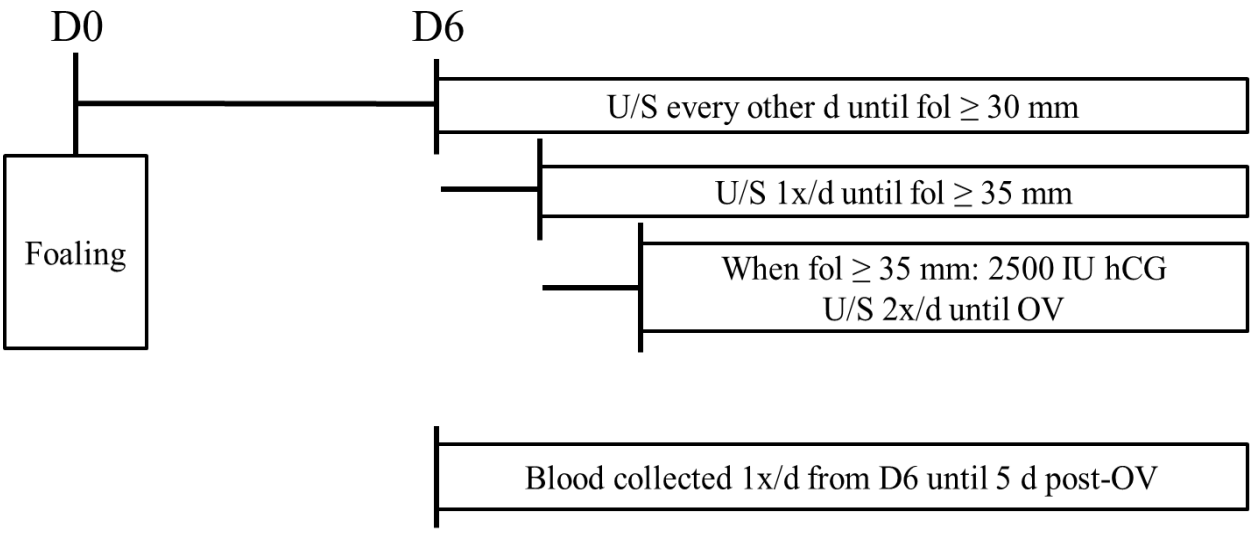

Figure 2.4 Procedural time line of mares left allowed to cycle normally post-foaling as untreated controls (CON)

Blood Collection and Analyses

Daily 10-mL blood samples were collected from all mares via jugular venipuncture at 0800 h into evacuated tubes beginning on d 6 and continued until d 5 post-ovulation. Blood samples were allowed to clot for 30 min at room temperature and then centrifuged at 2,500 x g for 20 min. Serum aliquots were stored at -20°C for future analysis. Serum samples were analyzed in one assay for progesterone (DSL-3900 ACTIVE® Progesterone Coated-Tube RIA kit, Diagnostic Systems Laboratories, Inc., Webster, TX). Assay sensitivity was 7.21 pg/ml and the intra-assay CV was 6.2%. This assay was previously validated in the equine and conducted according to the manufacturer protocol (Schmidt, 2010).

Statistical Analyses

Data were analyzed using the GLM procedure in SAS (version 9.1, Cary, NC). Differences in treatments were evaluated by using ANOVA. The model included treatment to determine the following outcomes: the interval from start of treatment to ovulation, days from hCG administration to ovulation, hours from hCG administration to ovulation, days from initial $PGF_{2\alpha}$ administration to ovulation, diameter of follicle ovulated, incidence of double ovulation and the diameter of the follicle at hCG administration. A cutoff point of > 1 ng/mL was used to determine luteal function. Concentrations of P4 were not different for each mare from d 6 to ovulation; therefore, P4 samples were pooled and analyzed as a single mean between treatments. Data are presented as least squared means ± SEM. Significance was determined at $P<0.05$.

Results

Experiment 1

Three FA mares and one P+E mare ovulated before hCG administration. One FA mare did not respond to hCG administration and did not ovulate before the end of the experiment. Data for these 5 mares were excluded from the interval from hCG administration to ovulation analysis. Interval from the initiation of treatment to ovulation, interval from hCG administration to ovulation, and interval from $PGF_{2\alpha}$ administration to ovulation were shorter ($P<0.01$) in FA mares compared with P+E mares. No treatment differences ($P>0.05$) were detected for incidence of double ovulation, diameter of the follicle at the time of hCG administration, or diameter of the follicle at ovulation. Rate of synchronization was calculated both including and excluding the 3 FA mares and 1 P+E mare that ovulated before hCG administration. Excluding those 4 mares, synchronization rates at 2 and 4 d post-ablation did not differ ($P>0.05$). Although numerically

greater in the P+E treatment, no difference in synchronization rates was detected when these 4 mares were included (Fig. 2.5, Table 2.1).

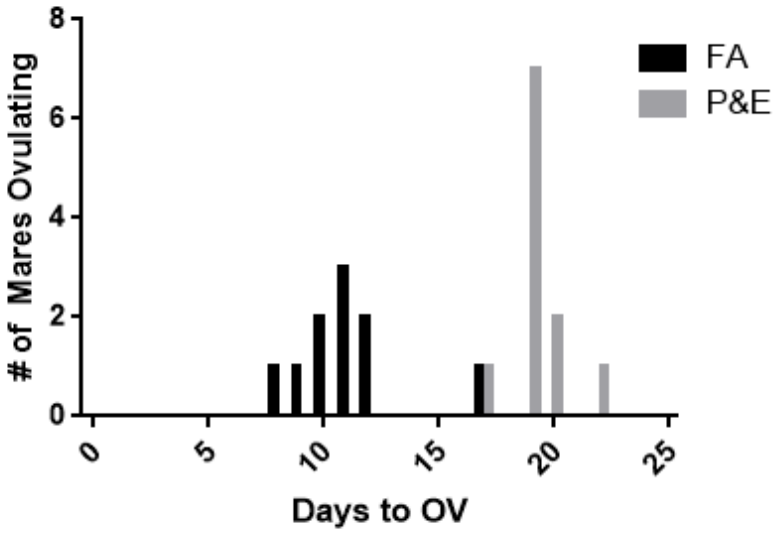

Figure 2.5 Distribution of the number of mares ovulating (OV) in response to treatment. Ovulation was synchronized by daily administration of progesterone and estradiol (P+E; n = 10) beginning on d 1 for 10 d with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) administered on d 10, and human chorionic gonadotropin (hCG) administered d 18 or all ovarian follicles > 10 mm were ablated transvaginally (FA; n = 9) on d 1 followed by administering $PGF_{2\alpha}$ twice 12 h apart on d 5 and hCG administered on d 11.

Table 2.1 Reproductive traits of mares in which ovulation was synchronized by either follicular ablation (FA) or a 10-d protocol of daily progesterone and estradiol (P+E) administration and follicles were monitored by transrectal ultrasonography until ovulation (OV) occurred.

	Treatment[c]	
Trait	FA[a]	P+E[b]
Treatment to OV (d)[f]	10.4 ± 0.4[d]	19.1 ± 0.4[e]
Interval from hCG to OV (h)[f]	28.8 ± 6.3[d]	55.5 ± 5.0[e]
Interval from $PGF_{2\alpha}$ to OV (h)[f]	6.5 ± 0.4[d]	10.1 ± 0.3[e]
Follicle size at hCG (mm)[f]	34.6 ± 2.2	32.9 ± 1.7
Follicle size at OV (mm)[g]	36.9 ± 1.7	33.9 ± 1.6
No. of mares synchronized within 2 d of first OV	6/7	7/9
No. of mares synchronized within 4 d of first OV	6/7	8/9
No. of mares synchronized within 2 d of first OV	6/10	7/10
No. of mares synchronized within 4 d of first OV	6/10	8/10
No. of mares that double ovulated[g]	2	2

[a]Ablation of follicles >10 mm followed by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) administration twice 12 h apart on d 5 and human chorionic gonadotropin (hCG) on d 11.
[b]Progesterone (150 mg) and estradiol (10 mg) administered once daily beginning on d 1 for 10 d followed by $PGF_{2\alpha}$ on d 10 and hCG on d 18.
[c]Values are listed as LSMeans ± SEM.
[d,e]Values within a row lacking a common superscript differ ($P<0.01$).
[f]n=7 for FA mares, n=9 for P+E mares.
[g]n=10 for FA mares, n=10 for P+E mares.

Experiment 2

Mare Reproductive Traits

Interval from foaling to ovulation was greater ($P<0.01$) in the FA treatment compared with controls (Figure 2.6). No treated differences ($P>0.05$) were observed for the interval from hCG administration to ovulation, incidence of double ovulation, or diameter of the largest follicle at the time of ovulation (Table 2.2).

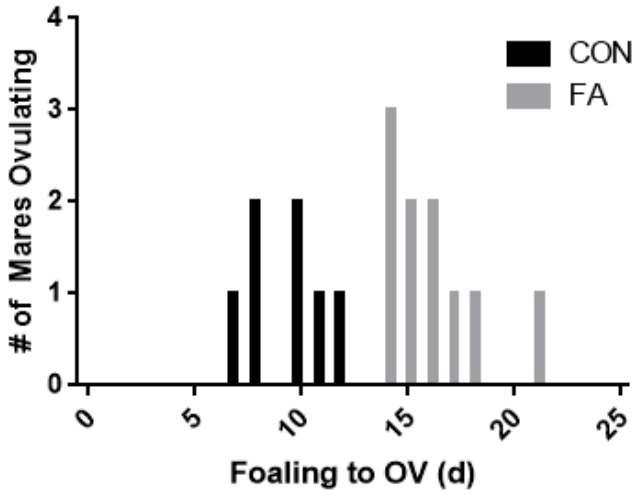

Figure 2.6 Distribution of the number of mares ovulating (OV) and days to ovulation after foaling (d 0) for mares whose follicles >10 mm were ablated on d 6 (FA, n=10), administered prostaglandin F2α ($PGF_{2\alpha}$) on d 11, and given human chorionic gonadotropin (hCG) once a follicle ≥ 35mm was detected. Control mares were allowed to cycle normally from foaling (CON, n=8) and given hCG when a follicle ≥ 35 mm was detected.

Table 2.2 Reproductive traits of early postpartum mares whose follicles >10 mm were targeted for removal by follicular ablation on d 6 post-foaling (FA) or allowed to cycle normally after foaling (CON) and monitored by transrectal ultrasonography until ovulation (OV) occurred.

	Treatment[c]	
Trait	FA[a]	CON[b]
Days from foaling to OV	15.8 ± 0.9[d]	10.0 ± 1.1[e]
Hours from hCG to OV	60.0 ± 6.5	48.0 ± 7.9
No. of mares that double ovulated	1	1
Follicle diameter at OV (mm)	43.7 ± 2.3	43.0 ± 2.8
Progesterone d 6 until OV (ng/mL)	1.1 ± 0.6[d]	0.2 ± 0.0[e]

[a]On d 6 postpartum, all follicles > 10 mm were targeted for removal via follicular ablation followed by (prostaglandin $F_{2\alpha}$) $PGF_{2\alpha}$ administration on d 11 and human chorionic gonadotropin (hCG) administration when a follicle ≥ 35 mm.

[b]Administration of hCG when a follicle ≥ 35 mm.

[c]Values are listed as LSMeans ± SEM; FA n=10, CON n=8.

[d,e]Values within a row lacking a common superscript differ (P<0.01).

Progesterone Concentrations

Average P4 concentrations from d 6 until OV were not different within each mare; therefore, P4 was pooled and analyzed as a single data point between treatments. Concentrations of P4 were greater ($P<0.01$) in FA mares when compared with CON mares (Table 2.2). Two mares in the FA group had elevated serum progesterone concentrations consistent with luteal tissue production. When data were removed for these two mares, no difference in average P4 concentrations was observed ($P>0.05$). No correlation was detected between average, baseline and median P4 concentrations and size of the follicle at ovulation ($P>0.05$).

Discussion

Current ovulation synchronization protocols dictate daily handling of mares and administration of exogenous hormones. The FA procedure has been utilized in other species to synchronize ovulation and offers the advantage of decreased handling and elimination of exogenous steroid hormone administration. Experiment 1 was conducted to determine if FA achieved comparable synchrony compared with P+E. Within a 4-d window from the initial observed ovulation, the overall rate of synchronization was 86% and 88% for the FA and P+E group, respectively. These results were consistent with previous findings for rates of synchrony within both treatments (Bergfelt et al., 2007). When data were included for the 3 FA mares and 1 P+E mare that ovulated before hCG administration, synchronization rates fell to 60% in FA mares, which was less than what was achieved with the P+E treatment; however, the difference was not significant. Reported synchronization rates for a 4-d window when using P+E range from 72% to 94% (Loy et al., 1981, Taylor et al., 1982) and this is comparable with the rate of synchrony achieved in this experiment. Acceptable and comparable synchrony between the two treatments was achieved when early-ovulating mares were excluded. When all mares were

included in the synchronization analysis, a greater variability of success was observed in the FA treatment. While ovulation synchronization was the objective of Experiment 1, it is important to note that the FA procedure shortened the interval from treatment initiation to ovulation when compared to P+E. Shortening the treatment to ovulation interval may make this procedure more attractive and warrants further investigation into the effects of narrowing the post-treatment time line.

Although stage of the estrous cycle at the beginning of treatment was recorded, it was not a significant factor in predicting whether or not synchronization would be successful and/or premature ovulations would occur. Compared with spontaneous follicular growth rates, it has been reported that follicles grow at a faster rate following an ablation procedure (Hinrichs et al., 1991, Cook 1995, Ginther et al., 2008) and this could have contributed to early ovulations observed before hCG administration in this experiment. Another contributing factor to this rapid ovulation could have been the presence of large follicles during the ablation procedure. When large follicles are targeted for ablation they can have confounding effects on the success of the procedure. These large follicles can regenerate follicular fluid to refill their antrum and regain or maintain dominance and go on to ovulate approximately 5% of the time (Bergfelt et al., 1994) or they can potentially develop luteal tissue and begin producing progesterone (Bergfelt and Adams, 2000). Luteinization of these large, and sometimes variable-sized follicles illustrates the necessity of $PGF_{2\alpha}$ administration following ablation, but perhaps a different administration interval than the one used in this study would be more beneficial to ensuring luteolysis. Generally, a 5-d interval from ovulation is required for prostaglandin receptors located within the plasma membrane of luteal cells to mature and respond to $PGF_{2\alpha}$ administration (Senger, 2005). In the FA synchronization protocol, only 4 and 4.5 days had passed before administration of

$PGF_{2\alpha}$. This timing was utilized in the current experiment based on previous reports of the use of this administration interval (Bergfelt et al., 2007). Lengthening this interval to $PGF_{2\alpha}$ administration could enhance the luteolytic response of the luteal cells and guarantee more successful luteolysis. Interval from initiation of treatment to ovulation in both the FA and P+E mares was shorter than previously reported (Bergfelt et al., 2007); however, in this study, the P+E protocol was adjusted to administer hCG on d 18 versus d 20 to follow closely more recent reports of this synchronization protocol (Loy, 1981, Mottershead, 2011), which may explain the shorter interval in the P+E treatment in this experiment.

Size of the follicle at the time of hCG administration is a critical component to the success of the ovulation-inducing agent. The administration of hCG is recommended when a follicle is > 35 mm in size, which will generally induce ovulation by 24 to 48 h post-administration (McCue, 2009). Interval from hCG to ovulation was within the predicted and acceptable window for the FA mares and was slightly longer than expected in the P+E mares. This longer interval may have been the result of the larger follicle size at the time of hCG administration in the FA treatment. Although close, neither group averaged a 35-mm follicle before administration of hCG. Set protocols for both treatments were followed to more closely follow current protocol directions. Administration of P+E has been shown to have a negative influence on circulating gonadotropin concentrations in mares (Evans et al., 1982). Although not measured in this study, decreased FSH or LH concentrations could have contributed to the slower follicular growth and longer interval from hCG administration to ovulation. To enhance the success of this protocol, more intensive monitoring may be employed to postpone hCG administration until the targeted follicle size of 35 mm has been reached.

Ovulation before hCG administration was observed in several FA mares and was potentially the result of incomplete removal of the follicle, which refilled and ovulated or perhaps became luteinized. Measurement of circulating progesterone concentrations would provide a more complete picture on whether the evacuated follicle(s) developed functional luteal tissue. In addition, measurements of all follicles that were ablated and ultrasound scanning of the mares in the days following the ablation procedure could provide useful data. Correlation data between size of ablated follicles and the predicted success of the procedure and the likelihood of a follicle to refill and ovulate or develop luteal tissue and begin producing progesterone would be beneficial. Although difficult on a large scale or in a commercial setting, more frequent ultrasound monitoring around the ablation procedure could provide useful insight into follicular dynamics following FA, particularly when used as a synchronization technique.

Experiment 2 assessed the practicality of using the FA procedure in mares to extend the postpartum interval to the initial ovulation. Maintaining a yearly foaling interval by foaling as early in the year as possible is advantageous in many areas of equine production. Breeding on the initial cycle post-foaling presents the challenge of decreased fertility associated with ovulations that occur too soon after parturition. This decreased fertility is partly a function of incomplete uterine involution, fluid sequestered in the uterine lumen, infection, inflammation, and other events, such as cervical and uterine trauma, at foaling (Zent, 2006). On average, with consideration to gestation length, a mare should ovulate and conceive within 25 days post-foaling to consistently produce a foal early in the next year. If ovulation occurs later than d 12 postpartum conception rates rise to 75%, which is considered an acceptable rate and would be comparable with ovulations during a conventional estrous cycle (Zent, 2006). Even when a mare may not naturally ovulate before d 12, lengthening the postpartum interval to ovulation would be

useful when a mare is going to be inseminated with shipped semen or required an ovulation-inducing agent to time breeding. Increasing the interval from foaling to ovulation also may be useful when a mare experiences mild dystocia during parturition. A few additional days before breeding would be beneficial to cervical and/or uterine repair and involution. To accomplish this goal of breeding between d 12 and 25, it is necessary to delay the expected first post-foaling ovulation period by 10 to 14 d (Loy et al., 1980). One method commonly used is administration of $PGF_{2\alpha}$ 5 d after the initial ovulation is observed post-foaling, commonly referred to as short cycling. Short cycling can decrease the interval from the first to second ovulation by approximately 9 to 10 d; however, ovulation must first be detected to appropriately time $PGF_{2\alpha}$ administration. In experiment 2, the aim was to determine if FA could be used to delay the postpartum interval to ovulation. On average, CON mares ovulated by d 10 postpartum. As discussed previously, breeding on this ovulation could have resulted in below average conception rates. The postpartum interval from foaling to ovulation in the FA treatment was increased to almost 16 d, which indicates pregnancy rates may have been improved over CON mares while falling within the necessary window to maintain a yearly foaling interval.

Interval from hCG administration to ovulation in both treatments was highly variable and longer than anticipated. Considering hCG was administered to all mares at the target follicle diameter of 35 mm, and the size of the follicle at ovulation was similar between treatments, a more consistent response was expected. Although not significant, the interval from hCG to ovulation was longer in FA mares. Circulating P4 concentrations were analyzed from d 6 post-foaling until ovulation to determine if any ablated follicles luteinized. Two mares in the FA group developed anovulatory hemorrhagic follicles (AHF) that produced progesterone following the ablation of large follicles. Both of these mares had follicles > 40 mm in size when the FA

procedure was performed. Complications with ablation or aspiration of large follicles that either refill and grow to ovulate or luteinize have been reported (Bergfelt and Adams, 2000). Administration of $PGF_{2\alpha}$ on d 5 post-FA regressed the AHF in one mare but not the other.

Conclusions

In conclusion, the FA procedure synchronized mares with a similar success rate compared with P+E. In addition, and possibly more important, FA shortened the interval from treatment initiation to ovulation compared with the P+E protocol. The stage of the estrous cycle at treatment initiation and the uncertainty of the fate of targeted large follicles could create variability in the success of treatment and synchrony. Additional management-related benefits of FA include decreased labor associated with daily handling for many days and eliminating exogenous hormone treatment. The FA procedure is a viable alternative for synchronizing ovulation; however, limitations of its use can be appreciated as the level of training and specialized equipment needed may make this procedure unavailable to individual mare owners, smaller veterinary clinics or clinics lacking theriogenologists trained in this procedure.

The FA procedure also successfully lengthened the interval from foaling to ovulation compared with untreated controls, and attained ovulation in the necessary window to both optimize conception rates and allow for a yearly foaling interval. Further research is necessary to determine if delaying ovulation, and thereby optimizing the conditions surrounding conception following FA, correlates with higher conception rates. Additional investigation into the effects of both lengthening and shortening the treatment interval to ovulation utilizing FA is warranted.

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Chapter 3 - Review of Current Literature for Experiment 3

Introduction

Horses are monogastric herbivores that are naturally designed to consume forage that is high in fiber and low in digestible energy. Domestication, in some cases, has altered the natural feeding tendencies of horses through the inclusion of meal feeding and the addition of concentrate rations. Horses, in general, are unable to tolerate high amounts of carbohydrates given at one time, which are fairly common in conventional concentrate rations. They are able to tolerate, however, large amounts of fat in their diet, which provides a caloric dense ration without the associated problems of feeding a high energy ration consisting of carbohydrates (Lewis, 2005, Bush et al., 2001). A horse can safely tolerate up to 20% of its diet as fat (Lewis, 2005) and current fat-added feeds contain between 6 and 12% fat to supply a caloric dense ration without large amounts of carbohydrates. Because of this level of tolerance and the need for additional calories as a result of the performance activities of some horses, inclusion of fat in a horse's diet has become a common industry practice. Increased fat allows for greater dietary energy without negatively impacting the glycemic index as do traditional increases in cereal grains alone (Bush et al., 2001).

Following the determination that horses are tolerant of fat-added diets, research was conducted to evaluate the effects of fat inclusion in the rations on many different classifications of horses (Hiney and Potter, 1996, Davison et al., 1991, Scott et al., 1989). In performance horses or horses in heavy work, positive benefits, such as decreased energy requirements and more efficient dietary energy usage and increased performance and recovery have been observed with the inclusion of additional fat in the diet (Hiney and Potter, 1996). Increasing fat in the diet supplies a safer alternative for providing a more energy-rich ration to horses with a heavy

workload (Hambleton et al., 1980). Harkins et al. (1992) found that skeletal muscle glycogen stores and resting glucose levels were increased, and race times were improved when racehorses were fed a high fat diet. In addition to performance-related benefits for the working horse, research has shown a variety of systems in a number of species will respond in a positive manner to dietary fat supplementation, including reproduction. The following review will discuss the types and sources of fat supplementation and its effects on reproduction and bone metabolism, including benefits to the horse and potential areas where further investigation is needed.

Fatty Acid Metabolism and Sources

Earlier studies typically supplemented fat in the form of plant and vegetable oils without regard to the actual makeup of the fat being provided. Historically, the benefits of fat supplementation were generally attributed to the increased caloric content. Research also indicated however, a possible impact of certain fatty acids (FAs), in addition to the caloric value of a higher fat ration. More recently the focus has shifted to the specific type of fat, in the form of polyunsaturated fatty acids (PUFAs) and their relationship to growth, reproduction and performance.

These FAs are characterized as a long hydrocarbon chain with a terminal carboxylic group in the alpha position and a methyl group at the omega position. The further definition of a FA is influenced by the position and number of the carbon to hydrogen (C-H) bond along the hydrocarbon chain. When the hydrocarbon chain is comprised of the maximum number of C-H bonds with no double bonds present, the fatty acid is categorized as a saturated fatty acid. The FAs that lack the maximum number of C-H bonds and contain double bonds are considered to be unsaturated FAs. Furthermore, if the hydrocarbon chain has more than one unsaturated, or double bond, it is characterized as a PUFA.

A PUFA can be further characterized as either an omega-3 (n-3), omega-6 (n-6) or omega-9 (n-9) FA depending on the position of the unsaturated bond located nearest the omega end of the hydrocarbon chain. When the unsaturated bond is located between the third and fourth carbons from the omega end, the FA is classified as an n-3 FA. When the unsaturated bond is located between the sixth and seventh and ninth and tenth carbons, the FA is classified as an n-6 and n-9, respectively. Although many FAs are necessary for bodily processes, only alpha-linolenic acid (ALA, C18:3) and linoleic acid (LA, C18:2) are considered essential because they cannot be synthesized by mammals due to the inability of mammalian desaturase enzymes to cleave beyond the ninth carbon from the alpha end of the hydrocarbon chain (Gurr et al., 2002). Essential fatty acids are an integral component of phospholipids and cell membranes (Kinsella, 1991) and are critical for many body processes including growth, reproduction and vision and brain development (Gurr et al., 2002). These essential fatty acids can impact growth and reproduction due to their ability to influence steroidogenesis and some transcription factors controlling gene expression (Wathes et al., 2007). Prostaglandins, leukotrienes, thromboxanes, lipoxins, and resolvins are also metabolites formed from enzymatic conversion of fatty acids that can differentially influence a wide array of bodily functions (Das, 2006).

Metabolism of the n-3 and n-6 FAs occurs in the liver where they undergo alternate desaturation and elongation reactions (Fig. 3.1) Each of these respective pathways utilize the same desaturase and elongase enzymes, which leads to competition between the pathways for intermediaries and subsequent production. The first enzyme to act upon the fatty acids is Δ-6 desaturase, which converts LA to gamma-linolenic acid (GLA) and ALA to steroidonic acid. The action of Δ-6 desaturase is a rate-limiting step in each pathway, with preference for the n-3 substrate of ALA. If enough precursor is available, this could shift production to n-3 pathway

production (Bezard et al., 1994). Following desaturase activity, elongation is the next step in each pathway, converting GLA and steroidonic acid into dihomo-gamma-linolenic acid (DGLA) and eicosatetraenoic acid (ETA), respectively. These intermediaries undergo further desaturation by Δ-5 desaturase which converts DGLA to arachidonic acid (AA) and ETA to eicosapentaenoic acid (EPA) in the n-3 and n-6 pathways, respectively. An additional desaturation and elongation step converts EPA to docosahexaenoic acid (DHA) through decosapentaenoic acid (DPA) as an intermediary.

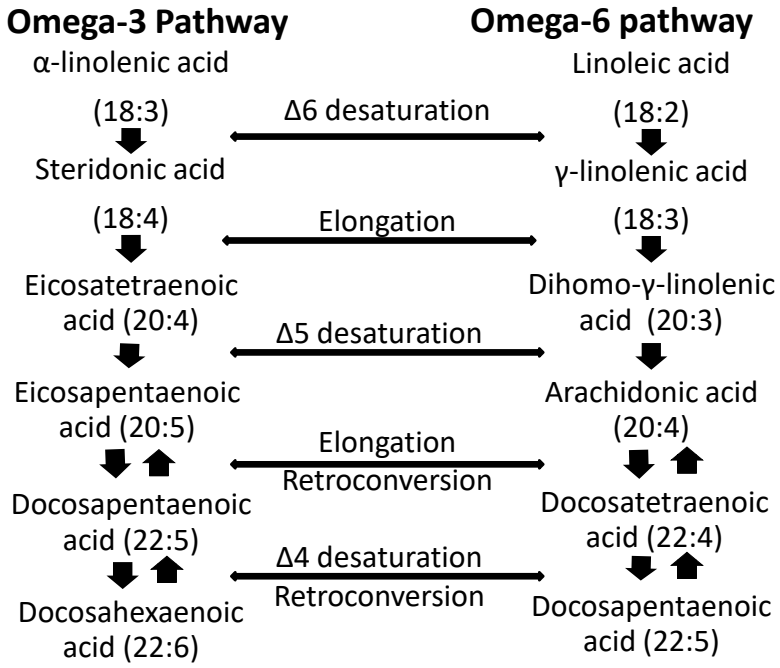

Figure 3.1 Essential fatty acid conversion pathway (adapted from Bezard et al., 1994)

FA Sources

Because LA and ALA cannot be synthesized by mammals, they must consume these fatty acids in their diet. Fresh forage, animal fat, nuts, and seeds such as flaxseed and linseed are common sources of ALA (Mueller and Talbert., 1988). Oils such as corn, sunflower, peanut, and soy oils contain high levels of LA (Carlier et al., 1991). Although not considered essential, EPA and DHA are physiologically important fatty acids as well, and while ALA can be converted to EPA and DHA, the conversion rate is unknown in the horse. In humans, the conversion rate is low (Larsson et al., 2004), indicating a need for direct supplementation of EPA and DHA. Sources high in EPA and DHA include marine species of fish (Mueller and Talbert., 1988) and to a lesser degree, plant sources such as algae (Doughman et al., 2007). By directly supplementing EPA and DHA, these n-3 FAs can be more rapidly incorporated into plasma and tissue by eliminating the rate-limiting step involving the desaturase enzymes in the conversion of ALA to EPA and DHA (King et al., 2008, O'Connor-Robinson, 2009).

FA Ratios

Research has focused not only on dietary inclusion of n-3 and n-6 fatty acids but also on their ratio because of their treatment effects on PUFA metabolites that influence pro and anti-inflammatory status in an animal. Humans typically consume a diet high in n-6 and low in n-3 FAs, resulting in diets that have a high n-6:n-3 ratio. Recommended ratios for human diets range from 4-10:1 (Albertazzi and Coupland, 2002). Horses typically consume a forage-based diet low in total fat but greater in n-6 FAs; however, the ratio of a typical horse diet consuming fresh forage is approximately 4:1 and is still within the recommended range for humans (O'Connor-Robinson, 2009). Preserved forage feeding rather than fresh grass has decreased the amount of n-3 FAs that horses would normally consume, so although the ratio is within recommended limits

for humans, these recommendations cannot be extrapolated to the equine. In addition, the relative importance of the ratio versus the amount of n-3s consumed by the horse has not been determined.

Early results demonstrated that supplemented EPA and DHA incorporate fairly rapidly into the horse's blood with increases detected in 3 d post-supplementation, reaching peak incorporation levels by 7 d (King et al., 2008). Although incorporation is rapid and a large number of commercial feed and supplements containing n-3 FAs are available, there are still no current equine feeding recommendations available. Many different supplementation levels have been investigated in prior studies, ranging from approximately 10 g of total n-3 to 30 g of EPA and DHA each. Due to their popularity and availability, more research is necessary to determine roles of specific FAs and the optimum rate of supplementation to address specific objectives.

Fatty Acid Effects on Eicosanoids and Inflammation

Dietary fatty acids will incorporate into the cellular membranes and impact the type of eicosanoids produced and used for intracellular signaling. Reproduction and inflammation are affected by PUFA metabolism through the role in the formation of arachidonic acid-derived eicosanoids, primarily prostaglandins, and their roles in the cyclooxgenase-1 and cyclooxygenase-2 pathways (Bagga et al., 2003). When n-3 PUFAs are supplemented in the diet, anti-inflammatory 3-series prostaglandins are favored (Abayasekara and Wathes, 1999). This process has the potential to have an impact on reproduction, injury, and disease. Other products that PUFAs influence that affect inflammation include cytokines, thromboxanes, and leukotrienes (Das, 2006). These products can also be anti-inflammatory or pro-inflammatory, and which products are produced is dependent upon available PUFA precursors (Patterson and Georgel, 2014). Generally, when n-3 PUFAs are more abundant in the diet, production of anti-

inflammatory products is favored and when n-6 PUFAs are more abundant, pro-inflammatory products are preferred.

Studies have been conducted in many species to determine the effect of n-3 supplementation on the inflammatory process. In humans, positive effects on patients with inflammatory conditions such as rheumatoid arthritis, osteoarthritis (OA), osteoporosis, and Crohn's Disease have been documented (Simopoulos, 1991). In addition, EPA improved the inhibition of cytokine formation and decreased pro-inflammatory adhesion molecules and degrading enzymes in patients with clinical rheumatoid arthritis (Adam, 2003).

The main focus in equine research are the potential benefits to horses with OA (Manhart et al., 2009). Factors involved in the inflammatory process that are typically assessed following supplementation are eicosanoids, primarily prostaglandins, cytokines, and leukotrienes. Hall et al. (2004) found that n-3 supplementation to healthy adult horses modulated the leukotriene B (LTB) inflammatory response. Fish oil supplementation greatly increased LTB5 and increased LTB4 as well. In horses with clinical OA, EPA and DHA supplementation decreased the synovial fluid white blood cell count and plasma PGE_2 concentrations when compared with controls (Hall et al., 2004). Plasma fibrinogen was also significantly decreased when analyzed from 30 d to 90 d after initiation of the treatment diet. Gibson et al. (1996) showed that horses with clinical OA had increased levels of PGE_2 within the synovial fluid but no differences in $PGF_{2\alpha}$ or LTB4. These data indicate the importance of PGE_2 as a potent mediator of inflammation and its usefulness as a measurement of joint disease and inflammation. When n-3 FAs are supplemented, preference for downstream metabolism through the n-3 pathway is preferred (Bezard et al., 1994); therefore, supplementation of n-3 FAs can impact this inflammatory mediator by shifting production from the n-6 pathway to n-3 products.

Supplementation of n-3 FAs has also been shown to decrease *in vitro* TNF-α, an important cytokine involved in the induction of inflammation (McCann et al., 2000; Dinnetz, 2009).

Circulating and synovial fluid concentrations of inflammatory mediators have been shown to be useful in determining the inflammatory status in the horse and these mediators can be affected by supplementation of n-3 FAs. While a normal young, growing horse may not experience a negative inflammatory status, there are several instances where inflammatory mediation may be necessary and useful. Young horses that are overfed or grow too rapidly may experience developmental orthopedic diseases (DODs) or epiphysitis associated with increased inflammation. Common medical treatments for these conditions involve invasive intra-articular steroid injections, long term administration of non-steroidal anti-inflammatory drugs (NSAIDs) and possibly invasive arthroscopic surgery (Sirin and Alkan, 2010). Long-term administration of NSAIDs may cause gastrointestinal issues, renal toxicity and inhibit healthy bone metabolism, potentially by altering prostaglandin synthesis that leads to uncoordinated bone healing (Knych, 2017); therefore, mitigation and/or prevention of inflammation in these instances would be useful. Dietary supplementation of n-3 FAs could potentially have a protective and beneficial effect on the young fast-growing, horse fed to exceed nutritional requirements.

Benefits of General Fat Supplementation on Reproduction

Positive effects of general fat supplementation on reproduction are well-documented in numerous species. In cattle, increased fat consumption has been linked to improved follicular dynamics (Hightshoe et al., 1991), shorter post-partum intervals before a return to estrus and increased conception rates (Mattos et al., 2002), and altered milk composition (Ashes et al., 1992). Supplemental fat has also been shown to increase circulating P4 (Staples et al., 1998) and

improve maternal recognition of pregnancy (Mattos et al., 2000) and embryo quality (Cerri et al.,2004).

Direct supplementation of PUFAs has also been shown to have positive effects in other species. Supplementation of n-3 FAs decreased circulating $PGF_{2\alpha}$ (Mattos et al., 2004) and n-6 supplementation decreased circulating $PGF_{2\alpha}$ (Cullens et al., 2004). Supplementation of n-6 FAs also decreased incidences of retained placentas, metritis and mastitis (Cullens et al., 2004). Supplementation of PUFAs increased follicle development postpartum and increased follicle size at ovulation (Lucy et al., 1993) which has been observed to hasten the first postpartum ovulation (Beam and Butler, 1997). In pigs, n-3 supplementation altered PUFA concentrations in the uterus, placenta and developing fetus (Brazle et al., 2009) and improved conceptus and piglet survival (Rooke et al.,2001). Positive effects of supplemental individual n-3s have also been indicated on reproduction in many species.

A previous study demonstrated that when mares were supplemented with additional fat during gestation and lactation, blood lipids were increased in the mare and foal, milk fat was increased, and foals had a higher rate of gain (Davidson et al., 1991). In addition, the authors found that there was a shorter postpartum interval to ovulation and a decreased number of cycles to conception (Davidson et al., 1991). Supplementation of n-3s has also been studied in the mare. Maternal DHA and EPA lengthened the postpartum interval to the first ovulation and increased the amount of time from development of a 35 mm follicle to ovulation during the first postpartum estrous cycle (Poland, 2006). These results of a longer postpartum interval to ovulation conflict with results reported by Davidson et al. (1991). A shorter postpartum interval to ovulation could have been attributed to differences in addition of fat in general vs. specific fatty acid supplementation of the Poland experiment. Schmidt et al. (2010) saw a decrease in the

amount of IGF-1 in the follicular fluid of n-3 supplemented mares around ovulation which the authors indicated might be responsible for the delayed interval to ovulation in the DHA and EPA supplemented mares. Improved endometrial scores and altered gene expression in the developing embryo following maternal n-3 supplementation in the mare indicates a positive effect on uterine environment and a possible mechanism for increased embryo survivability (Jacobs et al., 2015). Supplementation of n-3 FAs has also been shown to decrease the incidence of post-breeding endometritis (Brendemuehl et al., 2014). Supplementation of n-3 FAs can have an effect on a wide variety of reproductive areas and more investigation is needed to determine the role of n-3 supplementation in the postpartum mare and effects on the reproductive process.

Fatty Acid Supplementation Effects on Immunity

Research has shown that n-3 FAs incorporate into many different tissues and secretions, such as erythrocytes, muscle, and milk, and in the mare can be passed to the developing foal in utero and through suckling (Vineyard et al., 2010, Kruglik et al., 2005). There are a small number of studies examining the immunological effect of n-3 FA supplementation in the developing foal (Hodge et al., 2017, Kruglik et al., 2005, Duvaux-Ponter et al., 2004). Supplementation of n-3s has been shown to affect immunity; however, conflicting results indicate a need for further investigation. In healthy adult horses receiving n-3 supplementation, PGE_2, which is a potent mediator of inflammation, both decreased (Hall et al., 2004) and remained unchanged (Vineyard et al., 2010) in response to an immunological challenge.

Passive transfer of immunity is defined as the transfer of antibodies across the gut in the early neonatal period following the birth of the foal. Acquisition of humoral immunity is measured by the transfer of immunoglobulins from the dam to the foal through the milk (Jeffcott, 1972). Because of the characteristics of the mare's epitheliochoral placenta, immunoglobulins

cannot be transferred across the placenta. Immunoglobulins are large molecules that can only be absorbed intact for a short period of time, up to 24 h, in the foal's digestive tract (Galan et al., 1986). Humoral immunity is measured by IgG concentrations in the foal's blood and can be used to evaluate the success or failure of passive transfer. Humoral immunity is measured by IgG concentrations in the foal because it is an abundant immunoglobulin in the horse and has a high prevalence in colostrum and blood (Sheoran et al., 2000).

The results of the effect of n-3 FA supplementation on antibody production in the mare and subsequent milk secretions have been conflicting. Kruglik et al. (2005) reported higher IgG concentrations in colostrum at parturition from mares supplemented with EPA and DHA; however, no affect was seen at 12 or 24 h postpartum and no difference in foal serum IgG concentrations were detected at any time point. Stelzini et al. (2006) reported altered FA composition in milk and also mare and foal serum, but found that neither fish oil nor flax supplementation affected colostrum, milk, or foal IgG concentrations. In an additional study, Poland et al. (2006) found that there was no difference in IgG at parturition between mares supplemented with DHA alone, EPA and DHA, or corn oil. Possible differences in results could be due to supplementation length, amount of supplementation and IgG subtypes measured. The conflicting data suggest a need for further evaluation of the effects of n-3 supplementation on IgG production and transfer from the mare to foal.

Fatty Acid Effects on Bone Metabolism and Density

In healthy bone metabolism, there is a constant cycle of bone formation and resorption. Osteoblast cells are responsible for bone synthesis, while osteoclasts are responsible for bone resorption. Both processes are necessary for natural bone turnover; however, problems such as DODs can occur when bone resorption is greater than bone synthesis (Watkins et al., 2001).

Synthesis and absorption are measured and evaluated in the live subject through the use of various markers coinciding with bone metabolites.

Several markers, such as osteocalcin (OC) and bone-specific alkaline phosphatase (BAP), are used for the measurement of bone synthesis. Osteocalcin is produced by osteoblasts and released into systemic circulation, making it a useful noninvasive marker of bone metabolism (Lian and Gundberg, 1988). Concentrations of BAP can be a marker for bone formation and elevated activity may indicate bone disorders (Gomez et al., 1995). Telopeptides, such as n-telopeptides (nTX), cross-linked carboxyterminal telopeptide of type I collagen (ICTP) and C-terminal crosslinked telopeptide of type I collagen (CTX) are collagen fragments generated by bone resorption and concentrations in the blood can be useful indicators of increased bone turnover (Garnero, 2009). Another metabolite used to measure bone turnover is osteoprotegerin (OPG), which is a soluble cytokine produced by osteoblasts, and its associated tumor necrosis factor receptor (RANKL) that inhibits osteoclastogenesis (Yasuda et al., 1998). Bone mineral content (BMC) and bone mineral density (BMD) may also be useful measurements to determine bone health. Studies have evaluated these different markers of synthesis and resorption and the impact on these markers by certain factors such as n-3 supplementation.

Research in humans, animals, and in *in vitro* studies have shown promising results on the beneficial effects of n-3 supplementation and its relationship to improved bone health. Griel et al. (2007) reported a significant reduction in n-telopeptides (NTx), a marker of bone turnover, following an increase in ALA in the diet of human subjects. The concentrations of NTx were positively correlated with the inflammatory cytokine tumor necrosis factor-alpha (TNF-α) in all diets. In addition, BAP was not altered by increases in n-3 or n-6 supplementation. These results indicated bone synthesis was not altered by diet and that inflammatory cytokine expression was

correlated with increased bone turnover. Martin-Bautista et al. (2010) found that adult plasma FAs were increased after a year of supplementation with fortified milk enriched with EPA, DHA, and oleic acid. There were also significant increases in OC, OPG, RANKL, and OPG/RANKL. The OPG/RANKL ratio in bone is an important indicator of bone mass (Boyce and Wing, 2007). These data indicated there was increased bone synthesis, which conflicted with previous reports of no differences in bone synthesis (Griel et al., 2007) coupled with increased bone turnover following n-3 supplementation.

Increasing bone health and strength is an important welfare and economic concern in the poultry industry. Tarlton et al. (2013) found that supplementation of ALA via flaxseed provided in an ad libitum feed to laying hens decreased keel bone breakage, while increasing BMC, BMD, total bone volume, and trabecular bone volume. In contrast, Baird et al. (2007) saw no difference in bone characteristics when laying hens were supplemented with flaxseed formulated to provide differing ratios of n-6 to n-3 FAs.

A study in neonatal piglets, which were either low birth weight or very low birth weight, demonstrated that supplementation with AA and DHA increased BMC in the spine of low birth weight piglets and total BMC in very low birth weight piglets (Kohut et al., 2009). In addition, both weight groups showed a decrease in bone resorption related to control diets, whereas bone formation was unchanged. In a similar study in normal birth weight neonatal piglets fed at either a high or low supplementation level of AA and DHA, whole body and lumbar spine BMD was greater in the low group (Mollard et al., 2005). The low group also had decreased concentrations of NTx when compared with the high group and unsupplemented controls. Plasma OC and IGF-1, and *ex vivo* release of PGE_2 from the tibia were not different between groups (Mollard et al., 2005). The authors concluded low levels of supplementation provided a beneficial effect that was

not seen with higher levels. Positive effects at low levels of supplementation not seen at higher doses indicates an optimum level of supplementation may exist. Mollard and Weiler (2007) also reported that dietary AA and DHA decreased bone resorption but did not alter the circadian rhythm of bone turnover markers, NTx and OC in piglets. These results indicate positive effects of n-3 supplementation on bone health in neonatal and growing piglets.

Many studies have also used the mouse and rat as models for bone research. Bonnet and Ferrari (2010) supplemented mice with either EPA or DHA from 3 to 17 mo of age and determined that EPA and DHA increased trabecular bone volume at 8 mo of age. Supplementing EPA alone also improved cortical bone volume and thickness, reduced the age-related decline of OC, and increased circulating concentrations of IGF-1. Both the EPA and DHA diet increased serum leptin concentrations (Bonnet and Ferarri, 2010). Shen et al. (2006) supplemented adult male rats with either menhaden oil (n-3) or safflower oil (n-6). The n-3 supplemented group had the greatest BMC and cortical and subcortical BMD and also showed an increase in serum IGF-1, parathyroid hormone (PTH), vitamin D, and BAP. In addition, the n-3 group had decreased bone nitric oxide (NO) production and urinary calcium. The n-6 supplemented group had increased bone PGE_2 and serum pyrodinoline. These results indicate the n-3 group had greater bone density and increased bone synthesis when compared with the n-6 group, which had increased bone resorption.

In growing female rats, dietary DHA has been shown to increase tibia BMD, BMC, and decrease lipid peroxidation (Lukas et al., 2011). In addition, supplemented ALA increased serum OC concentrations, indicating positive effects of DHA on bone density and positive effects of ALA on bone synthesis (Lukas et al., 2011). Lau et al. (2009) supplemented LA to fat-1 mice that convert n-6 fatty acids to n-3 FAs. Twelve wk after initiating supplementation, femur n-3

concentrations were greater. They found that the high n-6:n-3 negatively correlated with BMC, and supplemented EPA and DHA positively correlated with BMC and peak load.

Maternal FA supplementation during gestation and/or lactation has also been shown to affect offspring bone formation. Li et al. (2010) supplemented artificially generated n-3 deficient mice pups that were separated from their dam at 2 d of age with either LA, LA and DHA, DPA, or DHA and DPA. Offspring at adulthood that were supplemented with DPA had decreased BMC and BMD. They found that DHA and total n-3 PUFAs strongly correlated to a positive increase in BMC. When rat dams were supplemented with AA and DHA during lactation, their offspring had enhanced spine BMC, tibia BMC and BMD, and whole body BMD as well as decreased OC. The rat dams had a decrease in the loss of whole body BMC and increased OC concentrations, indicating that n-3 supplementation enhanced bone density in the offspring while simultaneously protecting the dams from bone loss (Li et al., 2010). In a similar study, Fong et al. (2012) found that when rat dams were supplemented with DHA during pregnancy and lactation, their offspring had higher resting zone thickness at 3 wk of age. The resting zone is the innermost layer of the growth plate at the end of long bones (Abad et al., 2002). Male offspring also had an increased bone trabecular number at 3 wk as well as increased bone volume, increased osteoblast and decreased osteoclast numbers. In addition, expression of TNF-α, NFκB, and IL-6 were decreased in male offspring at 3 wk. No differences were detected beyond the 3 wk sampling period; however, rat pups were weaned onto rat chow that did not contain supplemental DHA. The authors summarized that perinatal DHA increased bone formation and resorption and increased bone mass in male, but not female offspring, and the beneficial improvements in bone health were not likely to continue into adulthood without continued supplementation (Fong et al., 2012). The authors also proposed a possible sex hormone effect on

the n-3 FA conversion and incorporation and its effects on subsequent metabolism that affected the bone changes. In another comparable study, rat dams were fed n-3, n-3 + n-6, or n-6 enriched diets. The n-3 + n-6 supplemented group had increased femur length and increased cortical cross section area and BMC of the rat dams (Korotkova et al., 2004). The authors proposed a beneficial effect of consuming a variety of different FAs. In the murine model, n-3 supplementation appears to influence markers of bone synthesis and remodeling and positively affect bone density and strength. Differences in results may be due to difference levels and lengths of supplementation. Although positive bone changes can be seen with n-3 supplementation, more research is needed to determine the optimal FAs that generate the greatest positive benefit and if these positive results can be replicated in non-murine species.

Although many studies evaluating the effects of n-3 FA supplementation on bone health and density have been conducted in other species, limited literature exists assessing these effects in the horse. No studies were identified investigating the effects of maternal periparturient supplementation and subsequent impact on the foal. Other traits, such as age and general nutrition in young, growing horses with and without history of DODs, have been investigated to assess their effects on bone health. When weanling horses were fed rations at a rate exceeding NRC recommendations, body weight and long bone growth was accelerated, possibly at the expense of skeletal development (Thompson et al., 1988). Excess dietary energy in growing horses has been shown to lead to an increased incidence of DODs (Glade and Belling, 1984). More research is necessary to determine if the positive effects of n-3 FAs, and more specifically EPA and DHA, can be observed in bone metabolism of the neonatal foal following maternal supplementation, especially those fed to exceed NRC requirements for moderate growth rates. An initial determination of whether supplementation can alter the measurements of bone health

in normal, healthy foals would provide a rationale for further investigation of a possible protective effect.

Summary

Supplementation of n-3 FAs has been demonstrated to alter circulating plasma FA profiles in the mare and foal. Positive effects on the reproductive traits of the mare have also been observed. In other species, addition of n-3s to the diet have had a positive impact on bone metabolism. Further research is necessary to determine if maternal supplementation of n-3 FAs during gestation and lactation impacts bone metabolism in the neonatal foal fed to meet their requirements.

Chapter 4 - Peripartum maternal DHA/EPA supplementation and reproductive traits in mares and foal bone metabolism

Abstract

Existing research is limited on foal bone health after peri-partum maternal supplementation of n-3 fatty acids. The objectives of the current research were to investigate the effect of maternal n-3 fatty acid supplementation on neonatal bone metabolism and mare reproductive traits. For this experiment, 17 pregnant stock-type mares were blocked by age, parity, and expected foaling date (EFD) and assigned randomly to either of two treatment diets: Control (CON; n=8, concentrate with no fat supplementation) or fat-supplemented (FS; n=9, concentrate supplemented with Gromega™; providing 13.475 g EPA/11.162 g DHA /d). Treatment began 8 wk before EFD. Blood collection for plasma fatty acid analysis began at treatment initiation and continued bi-weekly. Additional daily blood sampling from mares began once a 30-mm follicle was first detected post-foaling and continued until 5 d post-ovulation. Samples were analyzed for progesterone (P4) and insulin-like growth factor-1 (IGF-1). Mares were monitored by ultrasound beginning on d 4 and continued until ovulation was detected. Foal blood collection occurred at birth and weekly for 8 wk for later fatty acid and bone metabolite analyses. At 2 and 4 wk of age, synovial fluid (SF) was collected via arthrocentesis from the tarsotibial joint of each foal. Synovial fluid and plasma were evaluated for osteocalcin (OC), prostaglandin E_2 (PGE_2), and carboxyterminal telopeptide of type 1 collagen (ICTP). Synovial fluid samples also underwent simple cytological analyses. The study concluded for mares and foals at 8 wk. Data were analyzed as a repeated measures design using PROC MIXED of SAS. Plasma DHA and EPA concentrations were increased ($P<0.05$) in the mares and foals. Mare gestation length, concentrations of IGF-1 and P4, and postpartum interval to ovulation were

similar (P>0.05) between treatments. In addition, no differences were detected in foal plasma metabolites or SF PGE_2, or OC. The ICTP in SF was greater (P<0.05) in the FS treatment at wk 4 and SF total protein was greater (P<0.05) at both time points in FS foals. A correlation (r = 0.49) was detected (P<0.05) between concentration of SF and plasma ICTP. Maternal n-3 supplementation did not affect mare reproductive traits and had a minimal effect on markers of foal bone metabolism in healthy foals fed to meet their growth requirements.

Keywords: mare, foal, n-3 supplementation, bone metabolism

Introduction

Horses are monogastric herbivores that are naturally designed to consume forage that is high in fiber and low in digestible energy. Domestication has altered basic feeding principles of horses by including meal feeding and the addition of concentrates. Horses are unable to tolerate large amounts of carbohydrates, which are fairly common in conventional concentrate rations. In contrast, horses are able to tolerate large amounts of fat, which provides a calorie-dense ration without the associated complications of feeding a high energy ration consisting of carbohydrates (Bush et al., 2001; Lewis, 2005). Inclusion of fat in the diet has become a common horse industry practice and research has been conducted to determine the effects of dietary fat supplementation on many different classifications of horses, such as horses in hard work and breeding animals. Positive benefits for the horse in general, and more specifically for reproductive performance, have been seen with the inclusion of additional fat in the diet (Davison et al., 1991). More current research has investigated the effects of specific types of fat, in the form of polyunsaturated fatty acids (PUFAs). The omega-3 PUFA (n-3) and omega-6 PUFA (n-6), and their relative ratio, have been the focus of numerous studies related to growth, reproduction, and performance in the horse.

Because of the inefficient conversion of linoleic acid (LA) and α-linolenic acid (ALA) through the n-6 and n-3 pathways, respectively, the two n-3 fatty acids of primary interest are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Direct supplementation of EPA and DHA allows for more rapid incorporation into plasma and tissue by eliminating the rate-limiting step in the conversion ALA to EPA and DHA (King et al., 2008; O'Connor-Robinson, 2009). Supplemented EPA and DHA incorporate fairly rapidly into the horse's blood and their concentrations can be detected in 3 d, reaching peak incorporation levels by 7 d (King et al., 2008).

Dietary supplementation with n-3 fatty acids has been shown to impact steroidogenesis, oocyte development, and implantation in several species (Wathes et al., 2007). In the horse, n-3 supplementation lengthened the postpartum interval to ovulation and increased the amount of time from development of a 35-mm follicle to ovulation (Kouba et al., 2019). Concentration of insulin-like growth factor-1 (IGF-1) in the follicular fluid decreases in n-3 supplemented mares around ovulation (Schmidt et al., 2010). In mares supplemented with n-3 fatty acids, increases in estradiol-17β (E2) and progesterone (P4), as well as increased follicle and subsequent CL size and increased growth of the embryonic vesicle and embryo proper have been reported (Ravi et al., 2014).

In healthy bone metabolism, a constant cycle of bone remodeling occurs. Studies have evaluated different markers of synthesis and resorption of bone, and the impact on these markers after n-3 supplementation. Research in humans, animals, and also *in vitro* have shown promising results on the beneficial effects of n-3 supplementation with regard to improved bone health in adults and juveniles (Kajarabille et al., 2013, Lau et al., 2013). In humans, n-3 fatty acid supplementation reduced markers of bone turnover (Griehl et al., 2007) and increased plasma

fatty acids and synthesis and turnover of bone (Martin-Bautista et al., 2010). In livestock, supplementation of flaxseed (providing increased ALA) provided ad libitum in feed to laying hens decreased keel bone breakage, while increasing bone mineral content (BMC), bone mineral density (BMD), total bone volume, and trabecular bone volume (Tarleton et al., 2013). In addition, arachidonic acid (AA) supplemented to low birthweight piglets increased BMC in the spine, total BMC and decreased bone resorption (Kohut et al., 2009). Normal birth weight neonatal piglets fed small amounts of AA had greater whole body and lumbar spine BMD and decreased bone turnover markers (Mollard et al., 2005). Dietary AA and DHA decreased bone resorption but did not alter the circadian rhythm of bone turnover markers in pigs (Mollard and Weiler 2007).

Maternal supplementation during gestation, lactation, or both, also has been shown to affect offspring bone formation. Li et al. (2010) supplemented artificially-generated n-3 deficient mice pups that were separated from their dam at 2 d of age with LA, LA and DHA, docosapentaenoic acid (DPA), or DHA and DPA. As adults, the offspring that were supplemented with DPA had decreased BMC and BMD. They found that DHA and total n-3 PUFAs strongly correlated to a positive increase in BMC. When rat dams were supplemented with AA and DHA during lactation, their offspring had enhanced spine BMC, tibia BMC and BMD, and whole body BMD as well as decreased OC. The rat dams had a decrease in the loss of whole body BMC and increased OC concentrations, indicating that n-3 supplementation enhanced bone density in the offspring while simultaneously protecting the dams from bone loss (Li et al., 2010). In a similar study, Fong et al. (2012) found that when rat dams were supplemented with DHA during pregnancy and lactation, their offspring had higher resting zone (innermost layer of the growth plate) thickness at 3 wk of age. Male offspring also had an

increased bone trabecular number at 3 wk as well as increased bone volume, increased osteoblast and decreased osteoclast numbers. In addition, expression of TNF-α, NFκB, and IL-6 were decreased in male offspring at 3 wk. No differences were detected beyond the 3 wk sampling period; however, rat pups were weaned onto rat chow that did not contain supplemental DHA. The authors summarized that perinatal DHA increased formation and resorption of bone and increased bone mass in male, but not female offspring. The beneficial improvements in bone health were not likely to continue into adulthood without continued supplementation. The authors also proposed a possible sex hormone effect on the n-3 fatty acid conversion and incorporation and its effects on subsequent metabolism that affected bone changes (Fong et al., 2012).

In another similar study, rat dams were fed n-3, n-3 and n-6, or n-6 enriched diets (Korotkova et al., 2004). The n-3 and n-6 supplemented rats had increased femur length and increased cortical cross section area and BMC. The authors proposed a beneficial effect of consuming a variety of different fatty acids (Korotkova et al., 2004).

Although numerous studies evaluating the effects of n-3 FA supplementation on bone health and density have been conducted in other species, no current literature exists assessing these effects in the horse, especially maternal peri-parturient supplementation. Effects of age and general nutrition on bone health and related developmental traits in the young, growing horse are well-documented (McIlwraith, 2004). When weanling horses were fed rations at a rate exceeding NRC recommendations, body weight and long bone growth was accelerated, possibly at the expense of skeletal development (Thompson et al., 1988). Excess dietary energy in growing horses has been shown to lead to an increased incidence of developmental orthopedic diseases (DODs) in growing horses (Glade and Belling, 1984).

Studies have shown n-3 supplementation impacts reproductive function in the mare and that n-3 supplementation may have an impact on bone metabolism in many species. More research is necessary to determine effects of n-3 fatty acids, and more specifically EPA and DHA, on bone metabolism in the neonatal foal after maternal supplementation. The objective of the current study was to determine if maternal DHA/EPA supplementation during late gestation and early lactation would alter reproductive traits in the mare. The second objective was to determine if supplementation would affect bone metabolism markers for bone synthesis and resorption in the healthy, normal foal, which may indicate a protective effect is possible in accelerated foal growth.

Materials and Methods

The current experiment was reviewed and approved by Kansas State University Institution Animal Care and Use Committee.

Animals and Treatments

Seventeen pregnant, stock-type mares and their resulting offspring were enrolled in this study. The mares ranged in age from 4 to 15 yr, weighed an average 541.7 ± 38.1 kg, and were categorized as either primiparous or multiparous. Treatment diets were initiated 8 wk before the expected foaling date (EFD). Four wk before the EFD, each mare was administered pre-foaling vaccinations and a broad spectrum anthelmintic. Mares were group-housed in 3 to 5 acre lots with some grass cover and ad libitum access to fresh water and a trace mineral supplement. Alfalfa hay was group-fed based on aggregate weight, and a concentrate (Horseman's Edge®, Purina Mills, St. Louis, MO) was fed individually in a 3 m by 3 m enclosure to ensure each mare had restricted access to individual portions. Diets were formulated to meet or exceed NRC

(2007) requirements for mares in late gestation and early lactation. Proximate analysis of the supplement and the fatty acid profile of the feedstuffs and supplement are provided (Table 4.1).

Table 4.1 Chemical analysis and fatty acid composition (expressed as % of total fat) of feedstuffs and on eicosapentaenoic acid (EPA)/docosahexaenoic acid (DHA) supplement fed to mares during late gestation and early lactation.

	Feedstuffs[a,b]		
Item[a]	Concentrate	Alfalfa Hay	Supplement[c]
DM, %	88.09	90.21	98.41
Crude Protein, %	13.9	18.57	0.73
ADF, %	10.31	33.23	13.24
NDF, %	23.21	41.97	16.03
Fat, %	6.07	2.59	39.20
Ash, %			
Ca, %	1.40	1.40	7.87
P, %	0.59	0.27	0.00
K, %	0.91	2.89	0.00
DE (Mcal/lb)	1.53	1.24	1.67
Total n-6:	49.89	19.88	1.56
Linoleic acid	49.89	19.88	1.56
Arachidonic acid	0.00	0.00	0.00
Total n-3:	5.37	22.99	29.03
Alpha-linolenic acid	5.35	22.66	1.43
EPA	0.02	0.00	13.75
Docosapentaenoic acid	0.00	0.00	2.46
DHA	0.00	0.33	11.39
n-6:n-3 ratio	9.29	0.87	0.05

[a]Values listed as means.
[b]Calculated on a DM basis by SDK Laboratories, Hutchinson, KS.
[c]Percentage total fat as reported by JBS United, Sheridan, IN.

Mares were blocked by EFD and assigned randomly within blocks to either of two treatment diets: (1) control (CON, n = 8) consisting of a concentrate (Horseman's Edge®, Purina Mills, St. Louis, MO) with no additional fat supplementation and (2) fat-supplemented diet (FS, n = 9) consisting of a concentrate (Horseman's Edge®, Purina Mills, St. Louis, MO) supplemented with 250 g Gromega® (JBS United Feeds, Sheridan, IN), which is a marine-derived EPA and DHA supplement that provided 13.475 g EPA and 11.162 g DHA per day. Diets were formulated to be isocaloric. Mares in the FS treatment began an acclimation period 5 d before the initiation of the experiment to slowly introduce the supplement into the diet in 50 g increments to reduce feed refusal. Before feeding each FS mare, the supplement was measured out and top-dressed on the concentrate. A small amount of water, approximately 250 mL, was then applied to the feed to bind the powder to the feed and the supplement was mixed thoroughly in the concentrate ration.

Three wk before EFD, mares were monitored for impending parturition by visual inspection and mammary secretion testing. Once parturition was imminent, mares were placed in foaling stalls and observed for parturition. Following parturition, mares remained in a stall for at least 24 h, and then were turned out in a different 3 to 5 acre pasture with other postpartum mares. Pre- and postpartum mares were housed separately for the duration of the study. Postpartum mares also were fed individually and buckets were hung at an appropriate height to prevent foals' access to the treatment diet. The procedural time line for all mares is included in Fig. 4.1. Treatment diets continued through 8 wk postpartum, at which point the study was concluded and both mares and foals were removed from the trial.

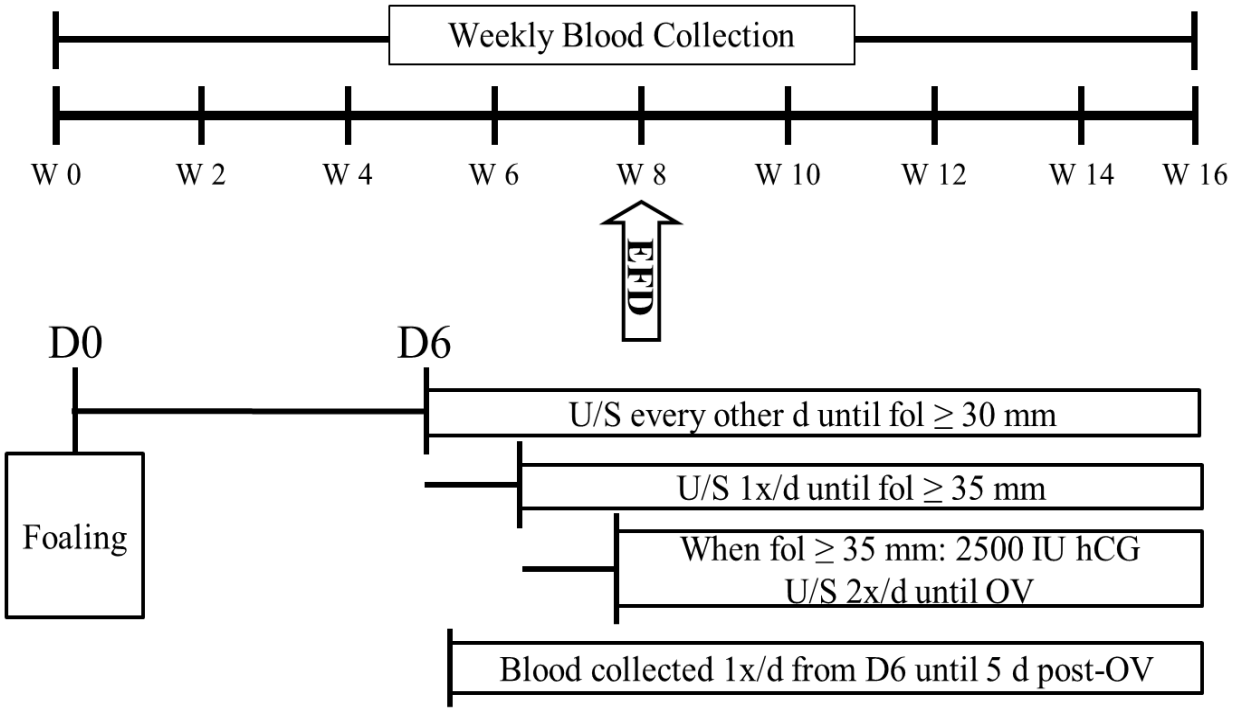

Figure 4.1 Procedural time line for all mares fed a concentrate ration (CON) or a fat-supplemented diet (FS)

Fatty Acid and Hormone Metabolite Analyses

Mare and Foal Blood Collection and Fatty Acid Analyses

Blood collection of mares via jugular venipuncture into 10-mL heparin additive Vacutainer® tubes began at the initiation of treatment (wk 0) and continued every 2 wk until the termination of the study. Following collection, blood samples were centrifuged at 2,500 x g for 20 min. Plasma aliquots were prepared and stored at -20° C for later analysis.

Blood sampling of foals via jugular venipuncture occurred at birth and weekly for 8 wk following parturition. Two 10-mL samples were obtained into Vacutainer® tubes, one with and one without heparin additive. Blood samples collected in serum tubes were allowed to clot at room temperature for 30 min while heparin additive tubes were stored at 5° C until centrifugation. After clotting was observed in the serum tubes, all blood samples were

centrifuged at 2,500 x g for 20 min. Plasma and serum aliquots were prepared and stored at −20° C for future analysis. Plasma fatty acid profiles from these blood samples were determined using gas chromatography techniques as described by Poland (2006). The procedural time line for all foals is included in Fig. 4.2.

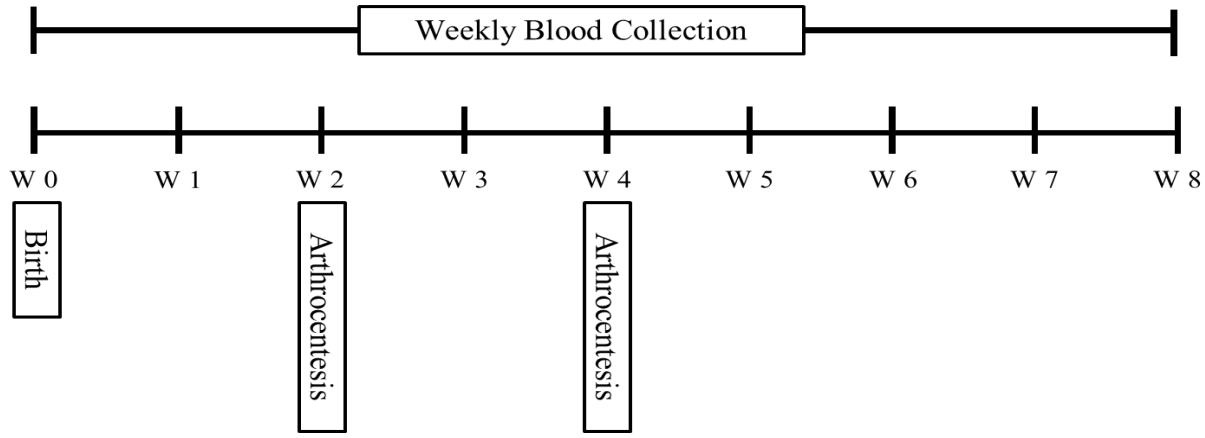

Figure 4.2 Procedural time line of suckling foals of mares fed either a control diet (CON) or a fat-supplemented diet (FS)

Mare Hormone Analyses

Additional daily blood sampling from each mare occurred once a follicle ≥ 30 mm was detected post-foaling via ultrasound evaluation, and continued until d 5 post-ovulation. Blood was collected via jugular venipuncture into 10-mL Vacutainer® tubes, allowed to clot at room temperature for 30 min, then centrifuged at 2,500 x g for 20 min. Serum aliquots were stored at −20°C for future analyses. Serum samples were analyzed for P4 (DSL-3900 ACTIVE® Progesterone Coated-Tube RIA kit, Diagnostic Systems Laboratories, Inc., Webster, TX) and IGF-1(DSL-5600 ACTIVE® IGF-1 Coated-Tube IRMA kit, Diagnostic Systems Laboratories, Inc., Webster, TX). Intra-assay CV, inter-assay CV, and sensitivity were 5%, 12%, and 2 ng/mL,

respectively for the IGF-I assay. Assay sensitivity for progesterone was 0.01 ng/mL and the intra-assay CV was 8.4%. All assays were previously validated in the equine (Schmidt, 2010).

Mare Ovarian Monitoring

Ovaries of each mare were monitored by transrectal ultrasonography every other day beginning on d 4 postpartum and continuing until the presence of a follicle ≥ 30 mm was first detected. Once a follicle ≥ 30 mm was observed, ultrasound frequency increased to once daily until ovulation was detected. Ovulation was characterized by the disappearance of the preovulatory follicle and subsequent corpus luteum formation. Gestation length and interval from foaling to first postpartum ovulation were calculated and the diameter of the follicle at ovulation was recorded.

Foal Bone Metabolism Markers

Plasma Samples

Foal plasma was analyzed for OC (MicroVue Osteocalcin ELISA Kit, Quidel, San Diego, CA) as a bone marker associated with bone synthesis. Plasma also was analyzed for ICTP (UniQ ICTP RIA, Orion Diagnostica, Immunodiagnostic Systems Inc, Scottsdale, AZ) as a marker of bone remodeling. In addition, concentrations of PGE_2 (PGE_2 ELISA Kit, ENZO, Farmingdale, NY) were analyzed because of its role in the inflammatory process and induction of bone resorption. Sensitivity for the plasma PGE_2 assay was 19.6 pg/mL and the intra-assay CV was 3.85%. The plasma OC assay had a sensitivity of 0.85 ng/mL and an intra-assay CV of 5.7%. Sensitivity for the plasma ICTP assay was 0.05 µg/L and the intra-assay CV was 8.0%. Assays were validated in equine plasma according to manufacturers' protocols.

Synovial Fluid Samples

At 2 and 4 wk of age, synovial fluid samples were collected via dorsomedial arthrocentesis from the tarsotibial joint of each foal's hock to measure intra-articular concentrations of bone markers. Foals in each treatment were selected randomly for the arthrocentesis to be performed either on the right or left tarsotibial joint at 2 wk. At 4 wk, the same joint was used for that foal. The procedure was conducted under standing anesthesia accomplished by administration of xylazine hydrochloride (0.4 to 0.8 mg/kg, IV, Anased®, Lloyd, Inc, Shenandoah, IA). Following routine aseptic preparation of the puncture sight on the hock, 2 to 4 mL of synovial fluid was obtained via dorsomedial arthrocentesis using a 20 g, 12 mm needle. Synovial fluid was separated immediately into 2 aliquots and placed on ice. One, 1 to 2 mL sample was placed in a heparin Vacutainer® tube and submitted to the Kansas State University Clinical Pathology Laboratory for cytology analysis of total nucleated cell count (TNCC) and total protein (TP). A second 1 to 2 mL aliquot was centrifuged at 450 x g for 5 min to remove any cellular contamination that could interfere with future analysis. These synovial aliquots were stored at −20°C for future analysis. The synovial fluid was analyzed for OC, PGE_2, and ICTP as previously described for the plasma analysis.

Sensitivity for the synovial fluid PGE_2 assay was 9.78 pg/mL and the intra-assay CV was 1.0%. The synovial fluid OC assay had a sensitivity of 0.85 ng/mL and an intra-assay CV of 8.6%. Sensitivity for the synovial fluid ICTP assay was 0.55 µg/L and the intra-assay CV was 5.05%. Assays were validated in equine synovial fluid according to manufacturers' protocols.

Statistical Analyses

A randomized complete block design was used for this study with mares assigned independently to the CON or FS treatment within blocks. Repeated measures were analyzed by

the MIXED procedure of SAS (Version 9.3, Cary, NC) with treatment, mare within treatment, day, and treatment x day included in a split-plot ANOVA. Treatment differences were tested by the mare within treatment variance (split-plot error), whereas effects of day and treatment x day were tested by the residual error (whole-plot error). Outcome repeated measures included mare and foal plasma FA profiles, mare serum IGF-1, P4, and E2 concentrations, and foal serum, plasma and synovial fluid bone markers, and foal synovial fluid cytology. Treatment by day means were separated within day by least squared differences (LSD).

Single time point data were analyzed by ANOVA and the model included treatment and block. Outcome variables included mare gestation length, interval from foaling to ovulation, and follicle diameter before ovulation. All data are presented as least squared means ± SEM and significance was determined at a $P<0.05$.

Results

Fatty Acids

Mare Plasma

A single mare foaled beyond 2 wk past her EFD. Her plasma FA data was truncated to 16 wk. Changes in mare plasma FA concentrations were evident during wk 2 after initiation of the treatment diets. Concentrations of LA did not differ ($P>0.05$) between treatments for the majority of the experiment, but were greater ($P<0.05$) in the CON compared with FS mares at 12 and 14 wk (Fig. 4.3). A significant day effect ($P<0.01$) was also observed in LA concentrations.

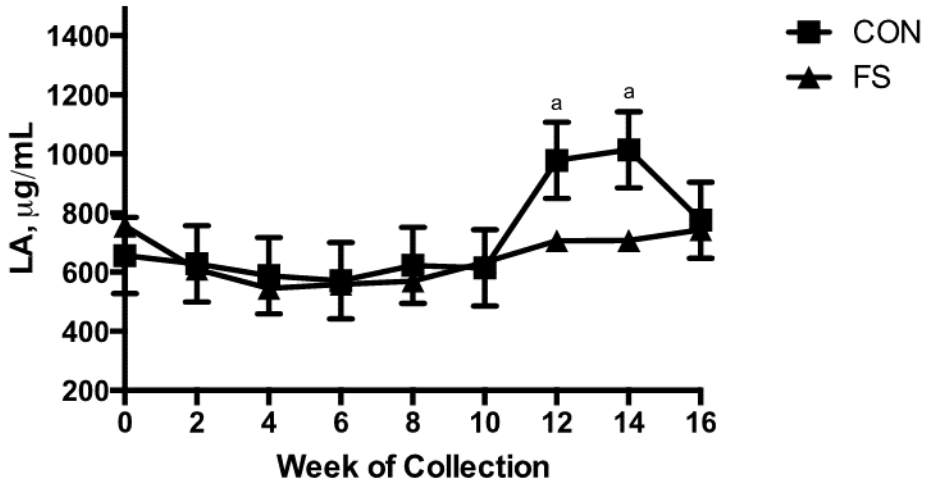

Figure 4.3 Mare plasma linoleic acid (LA) concentrations (LSMeans ± SEM) in response to dietary supplementation during late gestation and early lactation with a fish oil-supplemented diet (FS, n = 9) or a control diet (CON, n = 8). Parturition occurred between 8 and 10 wk. [a]P<0.05

Arachidonic acid concentrations were greater (P<0.01) in the FS treatment from wk 2 to 12, but did not differ (P>0.05) at 14 wk and were again greater in the FS treatment at 16 wk (P<0.01; Fig. 4.4). A treatment by day interaction (P=0.003), as well as a treatment (P<0.01) and day (P<0.01) effect, were observed in AA concentrations. Concentrations of ALA were comparable to LA, as they were similar for the majority of the trial, with a strong trend at wk 12 (P=0.058) and 14 (P=0.052) for increased concentrations in CON mares (Fig. 4.5). A day effect trend (P=0.057) was also detected.

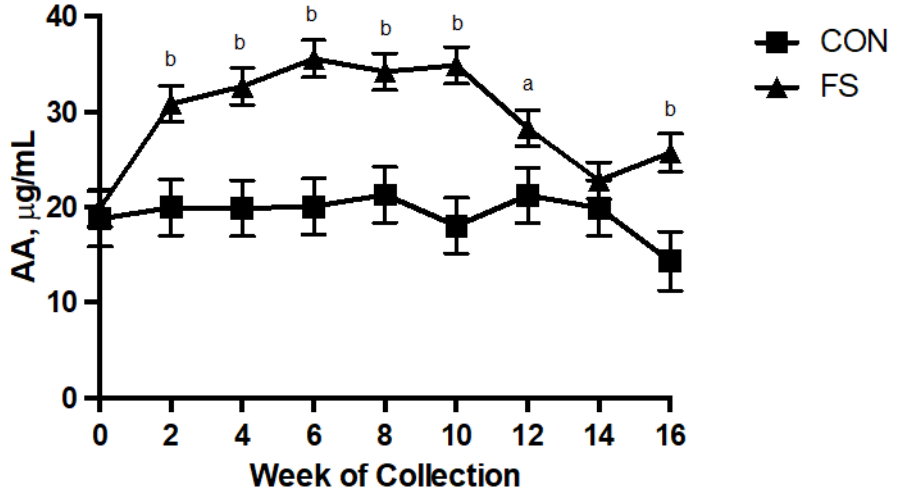

Figure 4.4 Mare plasma arachidonic acid (AA) concentrations (LSMeans ± SEM) in response to dietary supplementation during late gestation and early lactation with a fish oil-supplemented diet (FS, n = 9) or a control diet (CON, n = 8). Parturition occurred between 8 and 10 wk. [a]P<0.05, [b]P<0.01

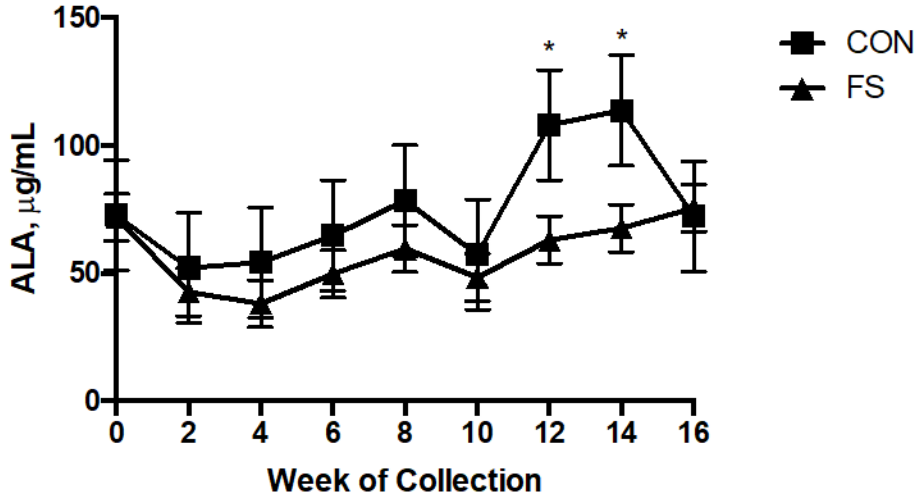

Figure 4.5 Mare plasma alpha-linolenic acid (ALA) concentrations (LSMeans ± SEM) in response to dietary supplementation during late gestation and early lactation with a fish oil-supplemented diet (FS, n = 9) or a control diet (CON, n = 8). Parturition occurred between 8 and 10 wk. [*]Denotes a trend P=0.06.

Plasma EPA was greater (P<0.01) in FS mares by wk 2 and remained greater throughout the trial compared with CON (Fig. 4.6). A treatment by day interaction (P<0.01), as well as an overall treatment (P<0.01) and day (P<0.01) effect, was observed. Plasma DPA was also greater (P<0.01) in FS mares compared with CON from 2 wk to 12 wk, similar (P>0.05) at wk 14, and again greater (P<0.05) in the FS mares at wk 16 (Fig. 4.7). A significant treatment by day interaction (P<0.01), as well as an overall treatment (P<0.01) and a day (P<0.01) effect, was observed.

Serum DHA concentrations were greater (P<0.01) in FS mares from wk 2 to 12 and at wk 16 (Fig. 4.8). At wk 14, a trend was observed (P=0.061) for greater DHA in FS mares. Treatment, day, and treatment by day interactions (P<0.01) were observed.

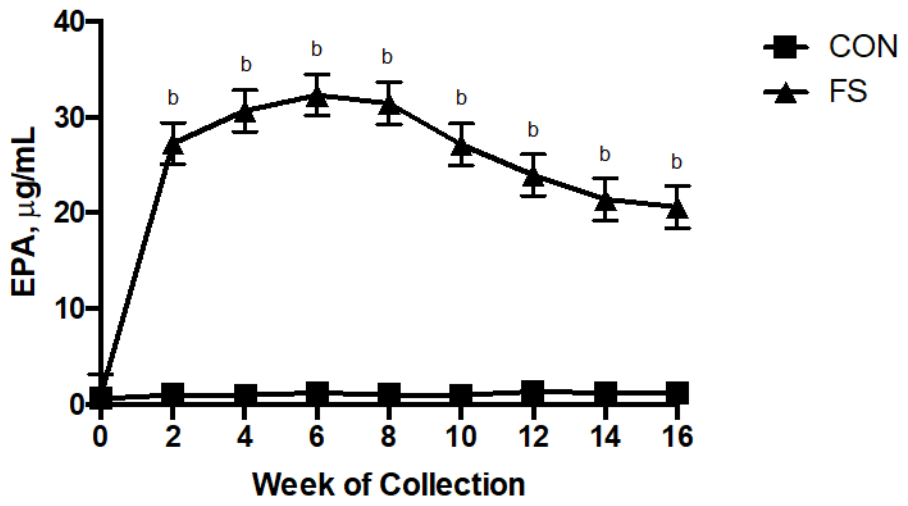

Figure 4.6 Mare plasma eicosapentaenoic acid (EPA) concentrations (LSMeans ± SEM) in response to dietary supplementation during late gestation and early lactation with a fish oil-supplemented diet (FS, n = 9) or a control diet (CON, n = 8). Parturition occurred between 8 and 20 wk. ^{b}P<0.01.

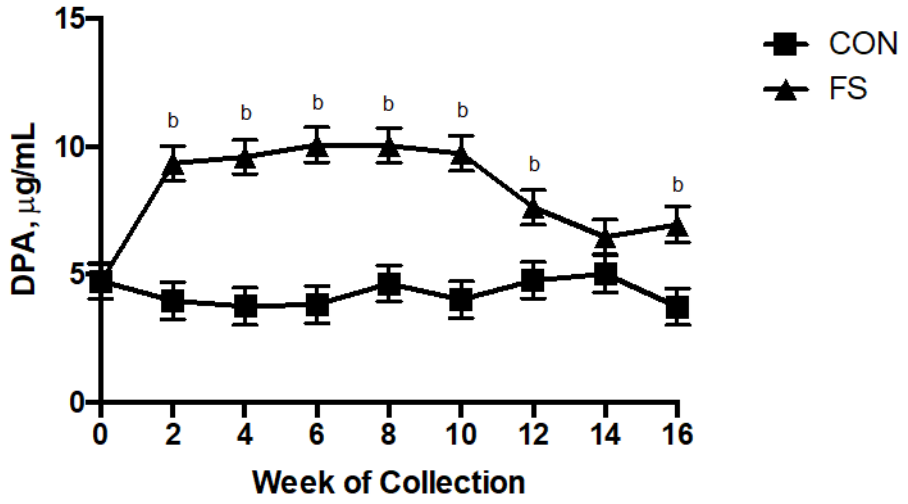

Figure 4.7 Mare docosapentaenoic acid (DPA) concentrations (LSMeans ± SEM) in response to dietary supplementation during late gestation and early lactation with a fish oil-supplemented diet (FS, n = 9) or a control diet (CON, n = 8). Parturition occurred between 8 and 10 wk. [a]P<0.05, [b]P<0.01.

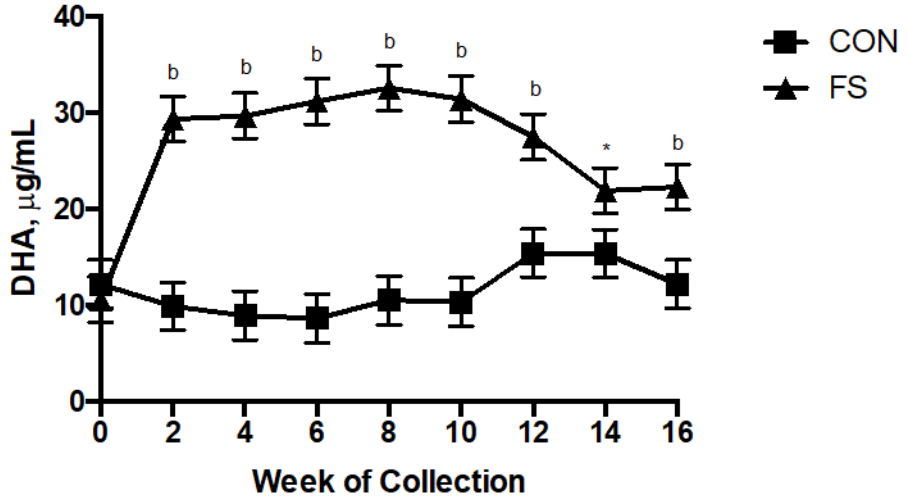

Figure 4.8 Mare plasma docosahexaenoic acid (DHA) concentrations (LSMeans ± SEM) in response to dietary supplementation during late gestation and early lactation with a fish oil-supplemented diet (FS, n = 9) or a control diet (CON, n = 8). Parturition occurred between 8 and 10 wk. [b]P<0.01. [*]Denotes a trend (P=0.06).

Foal Plasma

Concentrations of LA did not differ (P>0.05) between treatments (Fig. 4.9). A significant day effect (P<0.01) was observed in LA concentrations. Arachidonic acid concentrations were greater (P<0.05) in FS foals from wk 2 to 8 (Fig. 4.10). A treatment by day interaction was detected (P<0.01), as well as a treatment (P<0.01) and day (P<0.05) effect was observed for AA concentrations. Concentrations of ALA were variable throughout the study (Fig. 4.11). A trend (P=0.055) for greater concentrations at wk 4 and greater concentration (P<0.05) at wk 8 was observed in CON foals. A day effect (P<0.01) was also evident.

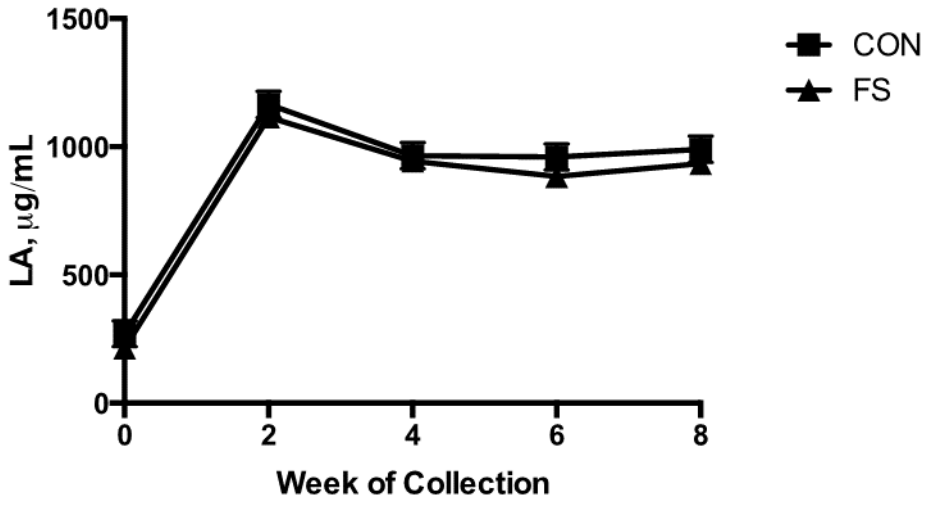

Figure 4.9 Foal plasma linoleic acid (LA) concentrations (LSMeans ± SEM) at birth (wk 0) and 2, 4, 6, and 8 wk of age when suckling mares were fed a control diet (CON, n = 8) or fish oil-supplemented diet (FS, n = 9) during late gestation and early lactation.

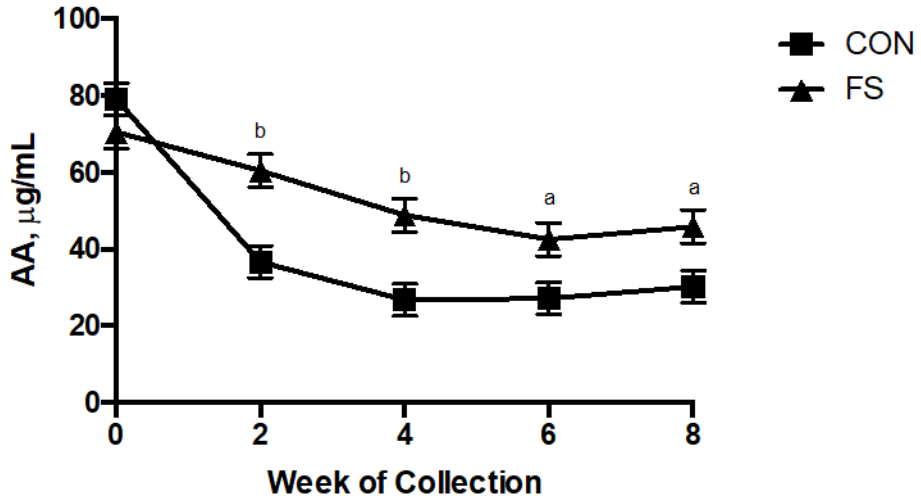

Figure 4.10 Foal plasma arachidonic acid (AA) concentrations (LSMeans ± SEM) at birth (wk 0) and 2, 4, 6, and 8 wk of age when suckling mares were fed a control diet (CON, n = 8) or fish oil-supplemented diet (FS, n = 9) during late gestation and early lactation. [a]P<0.05, [b]P<0.01.

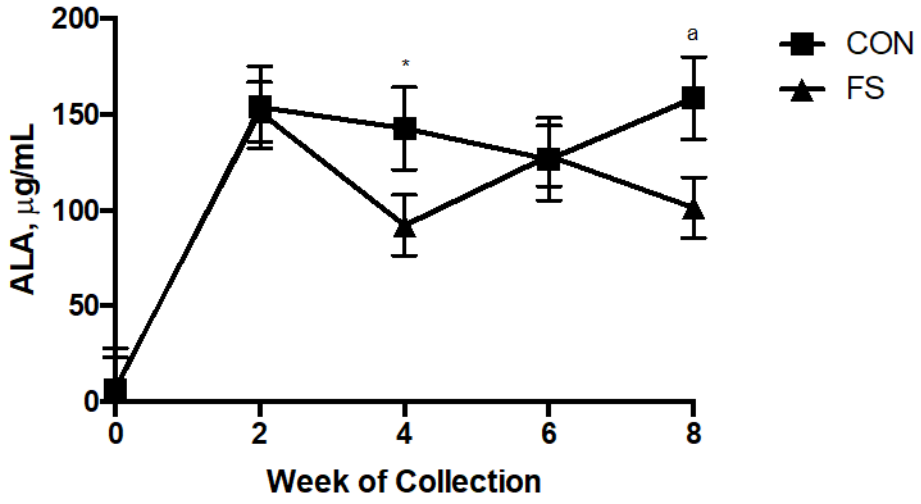

Figure 4.11 Foal plasma alpha-linolenic acid (ALA) concentrations (LSMeans ± SEM) at birth (wk 0) and 2, 4, 6, and 8 wk of age when suckling mares were fed a control diet (CON, n = 8) or fish oil-supplemented diet (FS, n = 9) during late gestation and early lactation. [a]P<0.05. * Denotes a trend (P=0.06).

Plasma EPA concentrations were greater (P<0.01) in the FS foals at birth (wk 0) and remained greater throughout the experiment compared with CON foals (Fig. 4.12). A treatment by day interaction (P<0.01) as well as a treatment (P<0.01) and day (P<0.01) effect were observed. Plasma DPA also was elevated (P<0.01) in the FS foals beginning at wk 2 and remained greater for the remainder of the experiment (Fig. 4.13). A treatment by day interaction was detected (P<0.01) in addition to a treatment (P<0.01) and day (P<0.01) effect.

Plasma DHA was increased (P<0.01) in FS foals from wk 2 to 4. A trend (P=0.07) was observed for greater DHA in FS foals at wk 6 with DHA concentrations again greater (P<0.05) by wk 8 (Fig. 4.14). A treatment by day interaction was detected (P<0.01) in addition to a treatment (P<0.01) and day (P<0.01) effect.

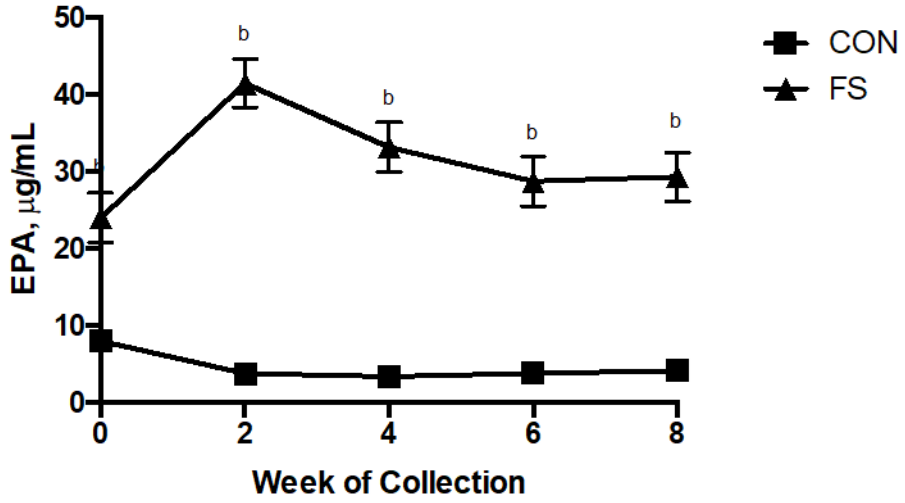

Figure 4.12 Foal plasma eicosapentaenoic acid (EPA) concentrations (LSMeans ± SEM) at birth (wk 0) and 2, 4, 6, and 8 wk of age when suckling mares were fed a control diet (CON, n = 8) or fish oil-supplemented diet (FS, n = 9) during late gestation and early lactation. [b]P<0.05.

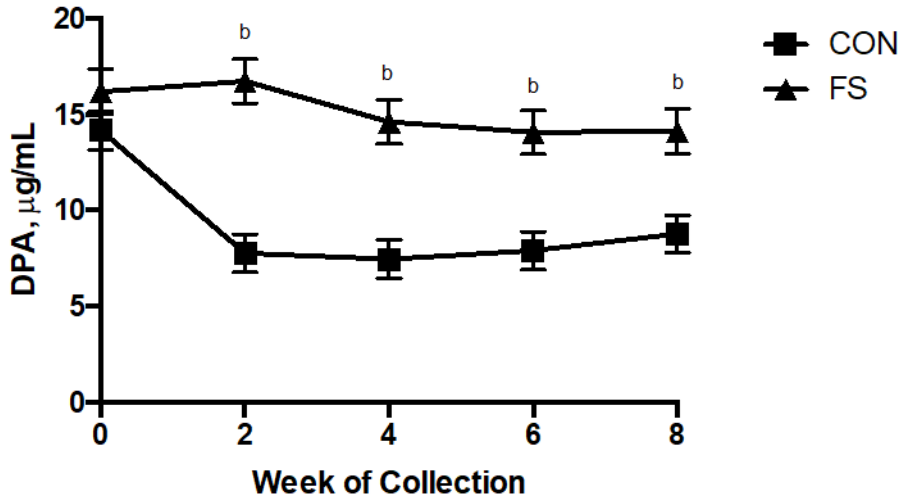

Figure 4.13 Foal plasma docosapentaenoic acid (DPA) concentrations (LSMeans ± SEM) at birth (wk 0) and 2, 4, 6, and 8 wk of age when suckling mares were fed a control diet (CON, n = 8) or fish oil-supplemented diet (FS, n = 9) during late gestation and early lactation. [b]P<0.01.

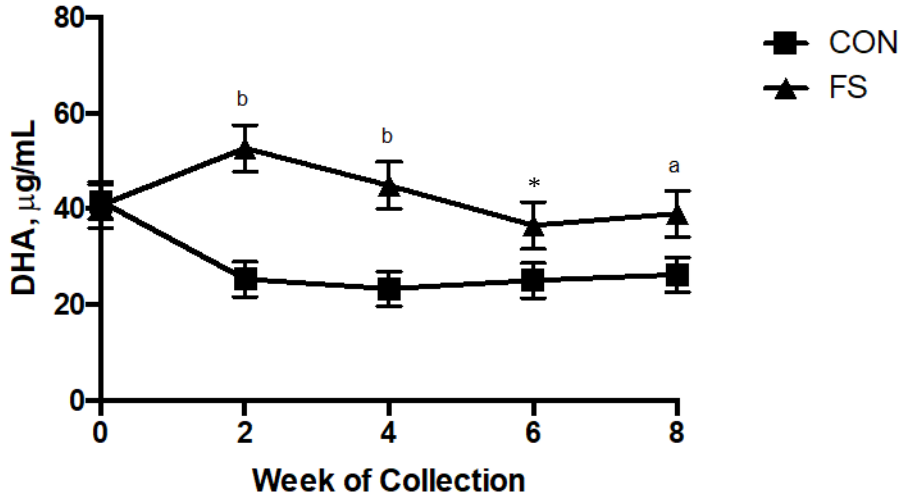

Figure 4.14 Foal plasma docosahexaenoic acid (DHA) concentrations (LSMeans ± SEM) at birth (wk 0) and 2, 4, 6, and 8 wk of age when suckling mares were fed a control diet (CON, n = 8) or fish oil-supplemented diet (FS, n = 9) during late gestation and early lactation. [a]P<0.05, [b]P<0.01. [*] Denotes a trend (P=0.06).

Mare Reproductive Traits

No differences (P>0.05) were observed in gestation length (346.4 ± 3.7 d for CON mares vs. 347.4 ± 3.3 d for FS mares), interval from foaling to initial postpartum ovulation (14.0 ± 2.5 vs. 17.3 ± 2.3 for CON and FS mares, respectively), or follicle diameter at ovulation (45.7 ± 1.5 vs. 47.2 ± 1.4 for CON and FS mares, respectively) between the FS and CON mares.

Mare Hormone Concentrations

No differences were detected between treatments for either serum IGF-1 (Fig. 4.15) or P4 (Fig. 4.16) concentrations during the peri-ovulatory period. As expected, a day effect was observed (P<0.01) for both IGF-1 and P4.

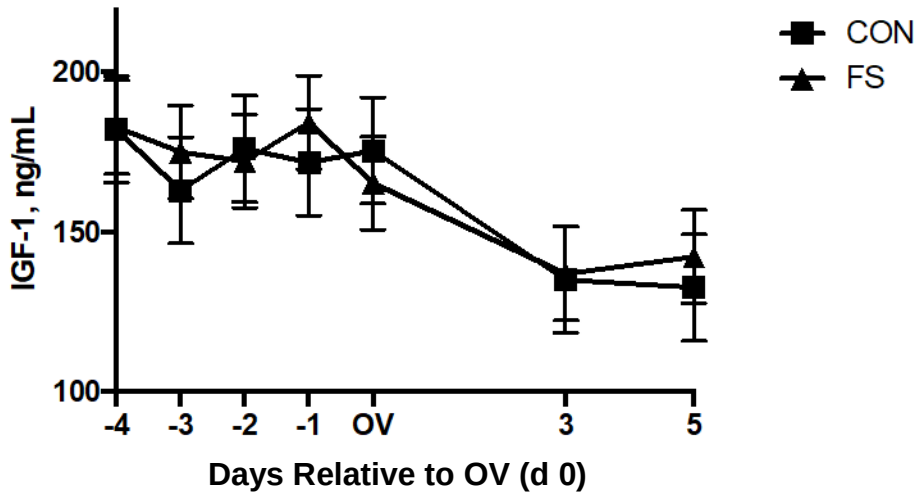

Figure 4.15 Serum insulin-like growth factor-1 (IGF-1) concentrations (LSMeans ± SE) during 4 d before first postpartum ovulation (OV), at OV, and on d 3 and 5 post-ovulation in mares fed a control diet (CON, n = 8) or a fish oil-supplemented diet (FS, n = 9).

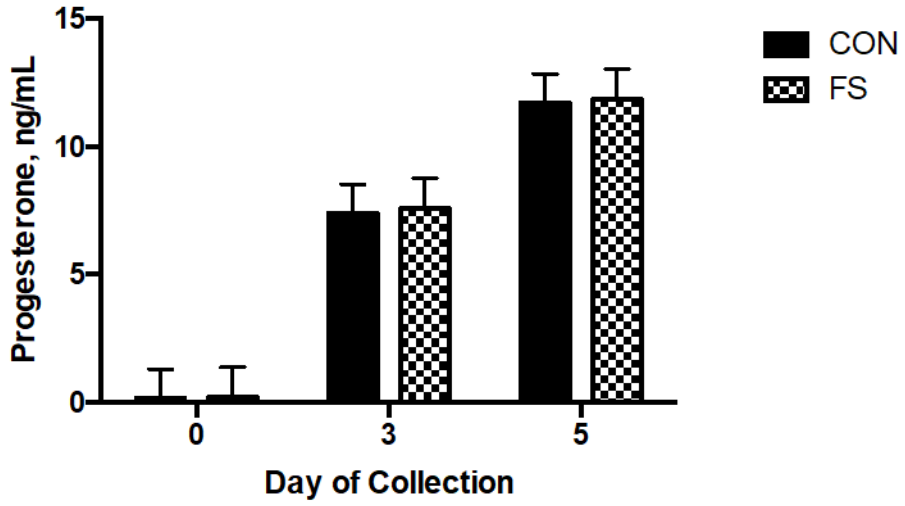

Figure 4.16 Serum progesterone concentrations (LSMeans ± SE) during the periovulatory period at first postpartum ovulation (d 0) and on d 3 and 5 post-ovulation in mares fed either a control diet (CON, n = 8) or fish oil-supplemented diet (FS, n = 9).

Foal Bone Metabolism Markers

Plasma

No differences (P>0.05) were observed in plasma concentrations of PGE_2 (Fig. 4.17), ICTP (Fig. 4.18), or OC (Fig. 4.19) between CON and FS foals. Concentrations of OC were less (P<0.01) in both treatments at wk 4 compared with wk 2.

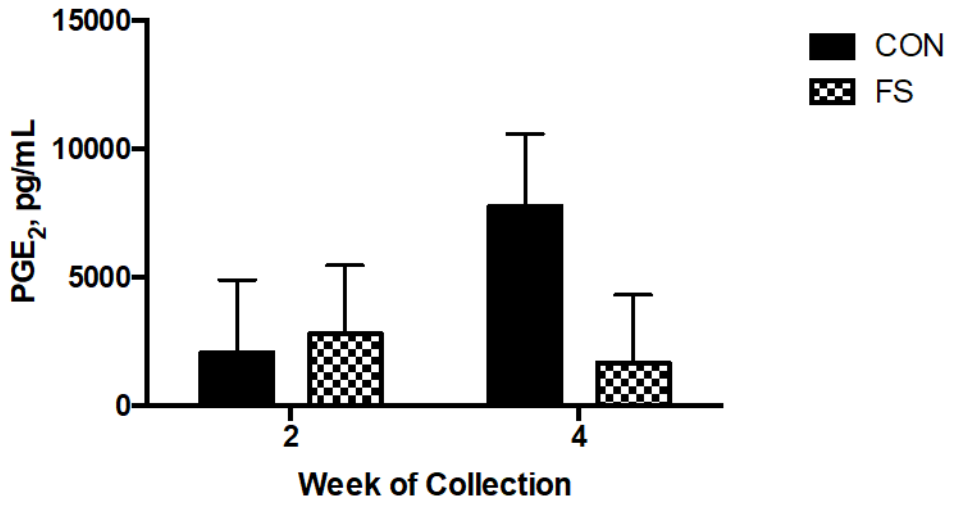

Figure 4.17 Plasma prostaglandin E_2 (PGE_2) concentrations (LSMeans ± SE) at 2 and 4 wk of age in foals suckling mares fed a control (CON, n = 8) or fish oil-supplemented (FS, n = 9) diet during late gestation and early lactation.

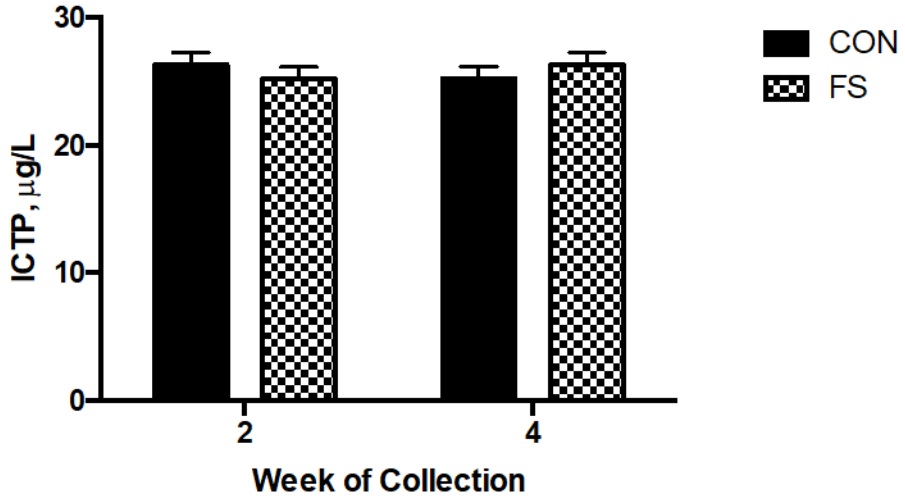

Figure 4.18 Plasma carboxyterminal telopeptide of type 1 collagen (ICTP) concentrations (LSMeans ± SE) at 2 and 4 wk of age in foals suckling mares fed a control (CON,n = 8) or fish oil - supplemented (FS, n = 9) diet during late gestation and early lactation.

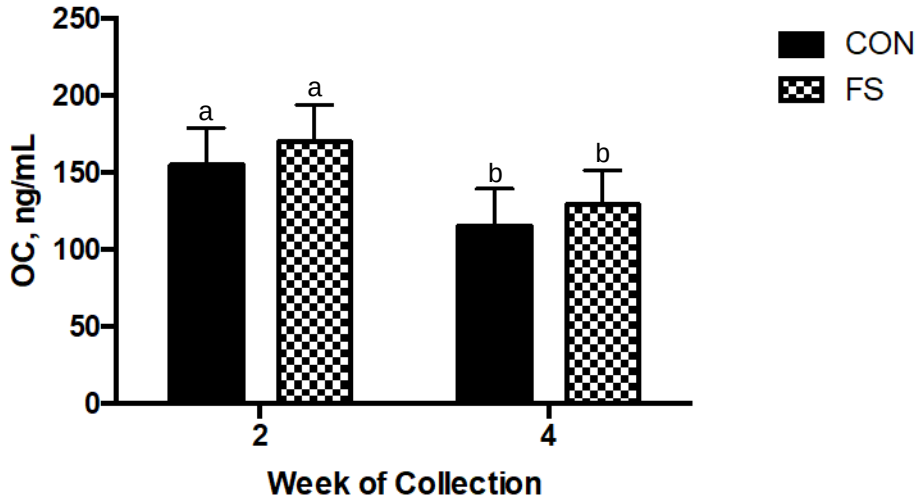

Figure 4.19 Plasma osteocalcin (OC) concentrations (LSMeans ± SE) at 2 and 4 wk of age in foals suckling mares fed a control (CON, n = 8) or fish oil- supplemented (FS, n = 9) diet during late gestation and early lactation.

Synovial Fluid

No differences (P>0.05) were found in synovial fluid concentrations of PGE_2 (Fig. 4.20) or OC (Fig. 4.21) between CON and FS foals. Concentrations of ICTP (Fig. 4.22) were greater (P<0.05) in FS foals at wk 4 compared with CON foals and a trend (P = 0.07) was detected for a treatment effect on ICTP concentrations in the FS foals.

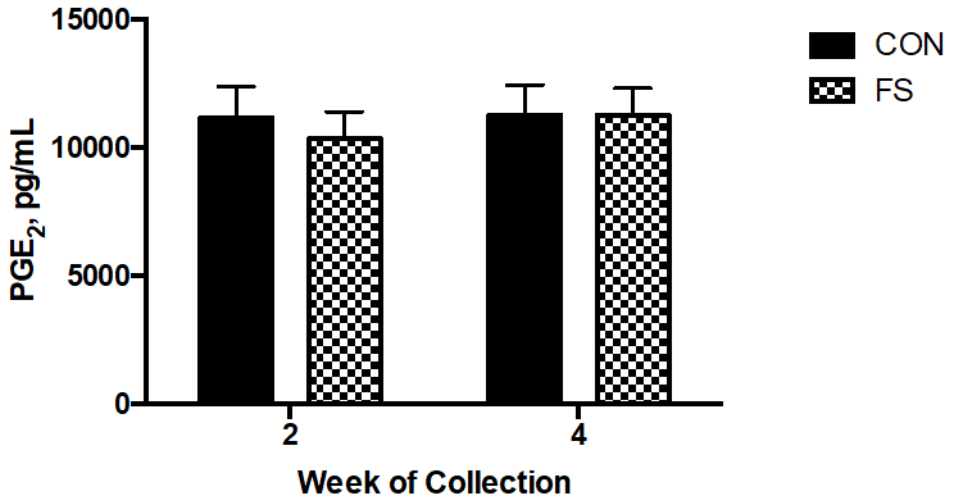

Figure 4.20 Synovial fluid prostaglandin E_2 (PGE_2) concentrations (LSMeans ± SE) at 2 and 4 wk of age in foals suckling mares fed a control (CON, n = 8) or fish oil-supplemented (FS, n = 9) diet during late gestation and early lactation.

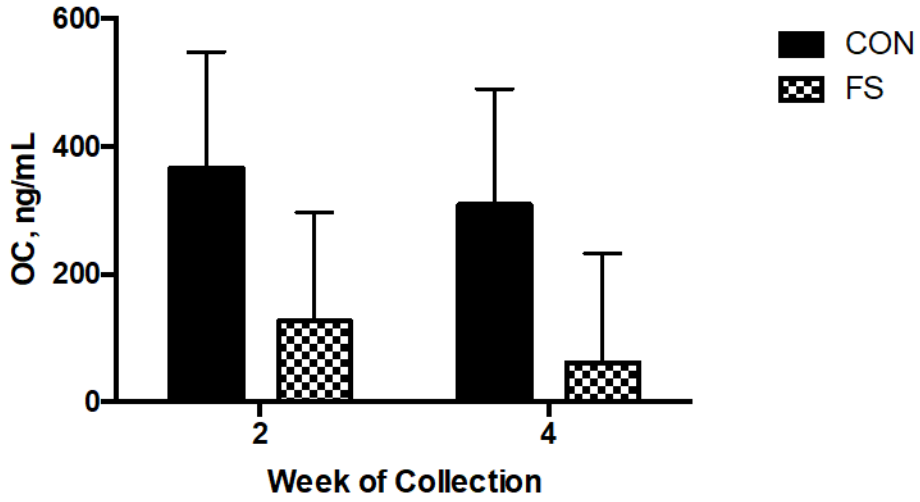

Figure 4.21 Synovial fluid osteocalcin (OC) concentrations (LSMeans ± SE) at 2 and 4 wk of age in foals suckling mares fed a control (CON, n = 8) or fish oil-supplemented (FS, n = 9) diet during late gestation and early lactation.

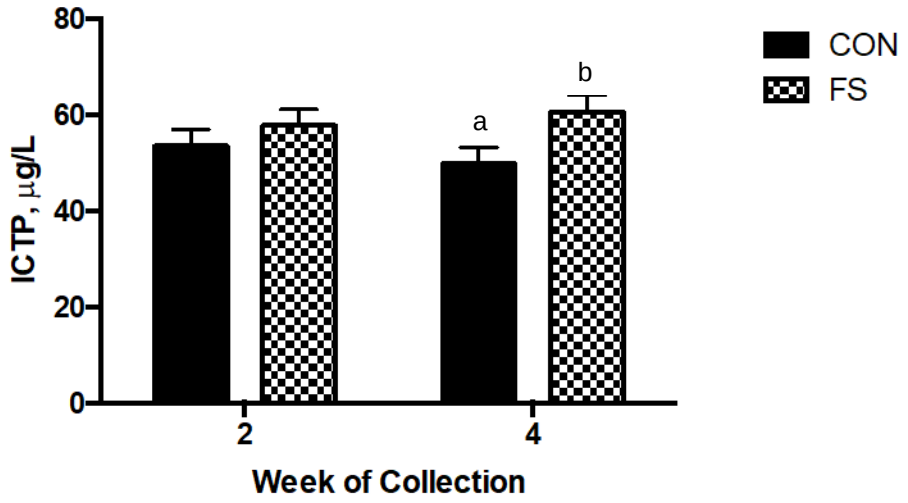

Figure 4.22 Synovial fluid carboxyterminal telopeptide of type 1 collagen (ICTP) concentrations (LSMeans ± SE) at 2 and 4 wk of age in foals suckling mares fed a control (CON, n = 8) or fish oil-supplemented (FS, n = 9) diet during late gestation and early lactation. Columns lacking a common superscript differ, P<0.05.

Correlations

No significant correlations (P>0.05) were detected between concentrations of PGE_2 (r = 0.04) and OC (r = 0.01) in the foal synovial fluid and plasma. A significant correlation (r = 0.49, P<0.05) was observed between ICTP concentrations in foal synovial fluid and plasma.

Synovial Fluid Cytology

Total protein was greater (P<0.05) in FS foals compared with CON at wk 2 and 4 (Fig. 4.23). An overall treatment effect (P<0.05) also was observed. No differences or effects (P>0.05) were observed in total nucleated cell counts between treatments (Fig. 4.24).

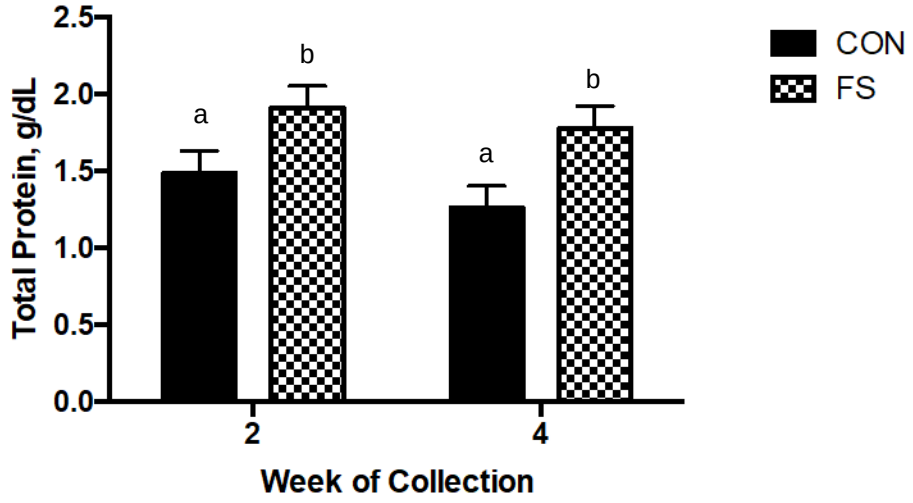

Figure 4.23 Synovial fluid total protein concentrations (LSMeans ± SE) at 2 and 4 wk of age in foals suckling mares fed a control (CON, n = 8) or fish oil-supplemented (FS, n = 9) diet during late gestation and early lactation. Columns within wk lacking a common superscript differ, P<0.05.

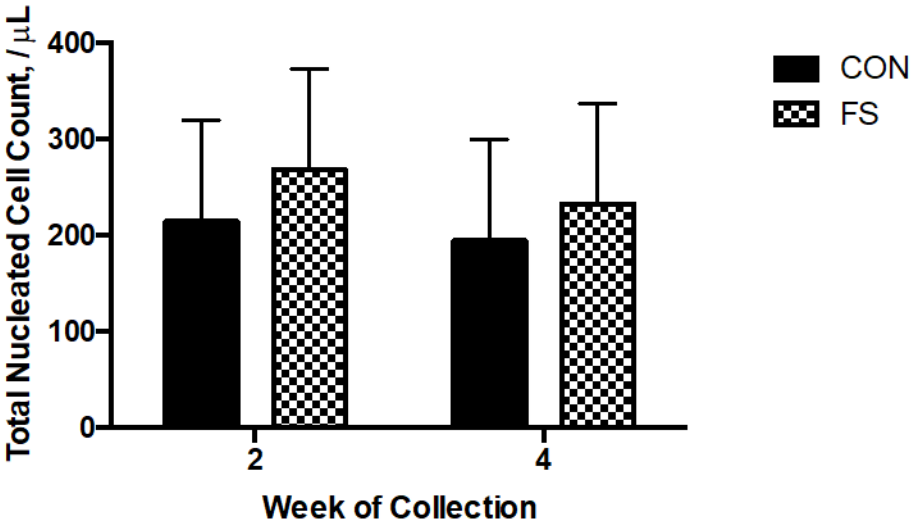

Figure 4.24 Synovial fluid total nucleated cell counts (LSMeans ± SE) at 2 and 4 wk of age in foals suckling mares fed a control (CON, n = 8) or fish oil-supplemented (FS, n = 9) diet during late gestation and early lactation.

Discussion

Plasma FA profiles in the equine have been well-characterized in general and the effects of n-3 supplementation on plasma FA concentrations also are well-documented. In this study, LA was found to be the most abundant PUFA in circulation, confirming previous studies (Bergero et al., 2002). The concentrations of LA were relatively stable and similar between treatments for the initial 10 wk of the experiment as well as the 16th and final wk; however, a significant increase in plasma LA concentrations was observed in CON mares at wk 12 and 14. A similar profile was also observed in mare plasma ALA. These results were expected as mares in both treatments were consuming the same diet and no differences in LA, AA and ALA were anticipated. By directly supplementing EPA and DHA in FS mares, additional substrate is provided for upstream conversion of ALA, AA and LA. Direct supplementation of these PUFAs can also decrease enzyme competition for substrate in the rate-limiting steps of upstream conversion (Williams and Burdge, 2006). Greater plasma concentrations of ALA and LA in CON mares at wk 12 and 14 could be explained by transition of seasonal grasses from dormancy into the growing season. Once mares foaled in early spring, they were moved to a different pasture that had more high quality smooth bromegrass availability. Mares typically foaled around the 8 to 10 wk mark and this could have coincided with increasing availability and new growth of forage to which the mares had access. Fresh green forages are a source of n-3 and n-6 fatty acids (Raes et al., 2004) and consuming fresh grass in addition to their concentrate and hay could have provided additional substrates of LA and ALA to the entire group, with the CON mares having less enzyme availability for down-stream conversion to EPA and DHA, thus a greater concentration of LA and ALA remained in circulation.

The effect of n-3 supplementation on plasma AA concentrations is variable in the literature. In the current study, AA generally remained greater in FS mares. Although AA is not typically a component of the n-3 pathway, previous research in our lab has shown both elevated plasma AA concentrations in FS mares (Poland, 2006, Schmidt, 2010) and similar concentrations between supplemented and control mares (Kruglik et al., 2005). Other authors have reported increased AA concentrations post-supplementation (Hall et al., 2004; King et al., 2008). Differences in the effect of supplementation on AA concentrations may be the result of differing amounts and duration of supplementation. Although not an efficient conversion, supplemental DHA could lead to additional AA production (Raz et al., 1998). In addition, as greater n-3 substrates are provided, there is a preferential shift in enzyme competition towards n-3 production and away from n-6 production (Abayasekara and Wathes, 1999). Dietary AA could undergo less conversion, and as uptake from target tissue preferentially takes up n-3s, greater amounts of AA could be left in circulation. No AA was provided in the concentrate or hay ration fed in either diet. Circulating AA could increase because of downstream conversion of LA, or the very inefficient process of upstream conversion of EPA and DHA metabolites or it may have been consumed in the fresh forage available to all mares.

Consistent with previous findings, supplementation of EPA and DHA increased plasma concentrations of these FAs in FS mares. Conversion of ALA to EPA and subsequently to DHA is not well defined in the horse; however, evidence indicates poor conversion rates (Hansen et al., 2002), which supports the concept that direct supplementation of EPA and DHA is a more efficient way to increase plasma concentrations of these fatty acids than supplementation of precursor FAs. King et al. (2008) found increases in plasma EPA and DHA concentrations as early as 3 d post-supplementation with peak concentrations at 14 d after initiating

supplementation in mares. In the current experiment, significant increases were observed for EPA, DPA and DHA by wk 2 of supplementation in FS mares; however, peak concentrations were not observed until wk 6 for EPA and DPA and wk 8 for DHA, followed by a gradual decline. The initial rise is apparently because of supplementation and the gradual decline in FA could have resulted from these FAs being removed from circulation and incorporated into target tissues (Vineyard et al., 2010). These mares also foaled during the weeks following peak concentrations of these FAs and the decline could be associated with the mobilization of these FAs in their milk. Evidence for an effect of maternal dietary n-3 supplementation on milk FA composition has been well documented (Kruglik et al., 2005, Stelzini et al., 2006, Kouba et al., 2019). The FA profiles of the mare's milk were not characterized in the current study; however, previous supplementation research supports increased n-3 FA concentrations in the milk. Increases in the foal's plasma n-3 FA profiles in this study and in previous research (Kruglik et al., 2005, Stelzini et al., 2006, Kouba et al., 2019) confirm the transfer of FAs both *in utero* and through the milk. Timing and peak concentrations of EPA and DHA were similar to previous reports in our lab using similar rates of supplementation (Schmidt, 2010, Kouba et al., 2019). When different supplementation amounts are used, peak concentrations can vary (King et al., 2008). Results have indicated a threshold level of FA uptake and incorporation into the blood, demonstrating the likelihood of an optimal supplementation rate with no further benefit observed following greater supplementation rates.

At birth, foals generally have little to no short and medium chain FAs in plasma, indicating these shorter chain FAs might be the preferential energy source for a neonatal foal and are taken up in the liver and are not abundant in circulation. Short and medium chain FAs have been demonstrated to be the preferential FA source in calves (Hocquette and Bauchart, 1999).

Placental transfer of FAs from the mare to the developing conceptus has been demonstrated in the horse (Stammers et al., 1987, Duvaux-Ponter et al., 2004). In this study, plasma FA concentrations in the foal followed similar trends to those seen in the mare with LA being the predominant plasma FA in the FS and CON foals. Similar to mares, foal plasma FA sharply increased after birth and was nearly identical between treatments during the 8-wk sampling duration. Foal AA concentrations were greater beginning at 2 wk and continued for the duration of the trial. Plasma concentrations were devoid of ALA at birth and sharply increased in both treatments by 2 wk. Although significant differences were observed at some time points, supplementation did not distinctly alter FA patterns or overall differences of ALA concentrations.

Consistent with the previous literature, circulating concentrations of EPA, DPA, and DHA were observed in all foals at birth. In FS foals, EPA was greater at birth, consistent with previous reports in our lab (Kruglik et al., 2005) and with other research in the foal (Stelzini, 2006). Blood sampling occurred before the foal suckling, thus FAs present in the foal plasma at birth provide evidence for placental transfer of these long–chain fatty acids (LCFAs). Concentrations of EPA, DPA, and DHA increased in the 2-wk sampling period following birth, confirming previous reports that foals are capable of both digestion and absorption of LCFA ingested from their dam's milk (Stelzini, 2006). Similar to mare plasma FA profiles, plasma concentrations of EPA and DHA showed peak concentrations followed by a decreasing trend that leveled out by wk 8. These data are consistent with previous reports (Kruglik et al., 2005, Stelzini, 2006, Kouba et al., 2019). Of note, although supplemented mares received DHA and EPA, DHA did not differ between foal treatments at the initial sampling period but was greater in FS foals from 2 wk on. In addition, peak concentrations of both EPA and DHA were greater in

FS foals compared with peak concentrations observed in the FS mares. Concentrations of EPA and DHA in the mare's milk have been reported to be concentrated, with values approaching 10 times the concentration in the plasma. Milk FA composition is not only determined by FA uptake from circulation by the mammary cell, but also by *de novo* FA synthesis in the mammary gland (Neville and Picciano 1997). When supplementing equal amounts of EPA and DHA to full-size horses and miniature horses, greater concentrations of these FAs are seen in the miniature horses (Furtney, 2009) and this could be the result of a smaller blood volume and decreased dilution of FA concentrations in circulation. Increased FA concentrations in the foal's blood may have occurred because of the difference in size and blood volume in addition to increased amounts of FAs ingested in the milk.

Previous studies of mare reproductive traits and hormone concentrations following n-3 supplementation have reported variable effects. In the current experiment, no differences were detected in gestation length, interval from foaling to the initial postpartum ovulation, or diameter of the follicle near ovulation. Our results contrast previously reported findings that n-3 supplementation increased the postpartum interval to ovulation in the mare (Poland, 2006). Poland (2006) found that n-3 supplemented mares underwent normal follicular growth following parturition, but stalled once a follicle > 35 mm was established, and the interval from development of a 35-mm follicle to ovulation was increased in the n-3 supplemented treatment. The author theorized that either a decrease in the availability of AA as a substrate for PGE_2 might have occurred or decreased IGF-1 in the supplemented group affected ovulation. Both PGE_2 (Martinez-Bovi and Cuervo-Arango, 2016) and IGF-1 (Donadeu and Pedersen, 2008) play crucial roles in follicle maturation and ovulation in the mare. A subsequent study found that follicular fluid concentrations of IGF-1 were decreased in mares supplemented with EPA and

DHA (Schmidt, 2010). Circulating plasma IGF-1 concentrations did not differ between treatments in the current study, but localized IGF-1 within the follicle was not evaluated and may have differed.

Reports of the effects of n-3 supplementation on P4 concentrations are also variable as both increases, decreases, and no differences following supplementation have been reported in horses and other species (Burke et al.,1997, Heravi- Moussavi et al., 2007). The mechanism for supplemental n-3 effect on P4 concentrations is unclear. Increased circulating P4 could be attributed to alteration of cholesterol uptake for P4 synthesis (Childs et al., 2008), alteration of transcription factors associated with P4 synthesis (Wathes et al., 2007), inhibition of COX-2 and increases in steroid acute regulator (STAR) protein (Wang et al., 2003), or simply decreased P4 clearance from circulation (Hawkins et al., 1995). Schmidt (2010) and Furtney (2009) both found increased circulating P4 concentrations in n-3 supplemented mares. In this study, no effect was observed on serum P4 concentrations in the mare during the peri-ovulatory period after 8 wk of supplementation. Inconsistency in reported effects on P4 concentrations may have resulted from a variety of factors including different supplementation amounts and duration, and different physiological states of the mares.

In healthy bone metabolism, there is a constant cycle of bone remodeling conducted by osteoblast and osteoclast cells. Bone synthesis and mineralization takes place rapidly after birth and then slows more closely to maintenance levels (Zeigler et al., 1974). Both resorption and synthesis are necessary for natural bone turnover; however, problems such as DODs can occur when bone resorption is greater than bone synthesis (Watkins et al., 2001). Studies investigating n-3 supplementation effects on markers of synthesis and resorption have reported promising, although variable, results in its relationship to improved bone health (Kajarabille et al., 2013). In

humans, n-3 supplementation has been shown to both significantly reduce bone turnover markers with no effect on markers of bone synthesis (Griel et al., 2007) and increase bone synthesis while increasing bone turnover (Martin-Bautista et al., 2010). Studies in livestock have indicated n-3 supplementation decreases bone breakage while increasing bone mineral content, density, and volume (Tarlton et al., 2013), and decreases markers of bone turnover (Mollard et al., 2005). Additional conflicting studies have reported no differences in bone characteristics following supplementation (Baird et al., 2007). Supplementation of n-3 FA in the murine model has been shown to increase bone synthesis (Shen et al., 2006, Lukas et al., 2011). Following maternal supplementation, offspring of n-3 supplemented dams had a strong correlation with increased bone mineral content (Li et al., 2010) and increased osteoblast number and decreased osteoclast number (Fong et al., 2012).

To this author's knowledge, the current experiment is the first to characterize maternal n-3 supplementation and subsequent effects on foal bone metabolism as measured by plasma and synovial fluid markers for bone synthesis and resorption. In contrast with several studies conducted in other species, the foal plasma and synovial fluid concentrations of PGE_2 and OC did not differ between treatments; however, there was a trend for elevated plasma ICTP concentrations in FS foals at wk 2 followed by greater concentrations by wk 4. These results are similar to a human study that found no changes in bone synthesis markers but increased bone resorption markers (Griel et al., 2007). Because retrieval of synovial fluid through joint arthrocentesis is an invasive procedure requiring specialized skills, the correlation between values of bone metabolites in the synovial fluid and concentrations in plasma were examined to determine if analysis of the blood would be an accurate indicator of bone markers in synovial fluid. No correlation was detected between PGE_2 and OC; however, a significant correlation was

observed between ICTP in the blood and synovial fluid, indicating plasma levels of ICTP may have limited use as a predictor of synovial fluid values. While significant, the correlation value was lower than typically associated with a strong correlation, thus indicating a low diagnostic value as a predictive indicator.

Foal synovial fluid also was submitted for cytological evaluation. Total protein in synovial fluid can be a useful measure of joint inflammation and TNCC can be a useful indicator of joint infection (Forsyth, 2018). No differences were detected in TNCC either between treatments or at different collection time points; however, a significant treatment effect was observed when comparing TP between treatments. The TP was surprisingly greater in foals nursing supplemented mares compared with controls. Due to the anti-inflammatory effects of n-3 supplementation, it was theorized n-3 supplementation would decrease TP in the FS treatment. Even though TP in the joint was greater in FS foals, no increases were observed in synovial fluid PGE_2 concentrations, indicating foals were not in a pro-inflammatory state. Although not investigated in the current study, n-3 supplementation has the ability to alter FA composition of the synovial fluid in humans (Wu et al., 2017). Although measurement of synovial fluid FA profiles would have been useful in the current study, limited sample volume prohibited further analysis. The effect of n-3 supplementation on the alteration of FA composition in synovial fluid of horses may provide evidence of its ability to alter the inflammatory status within the joint. Further investigation into different supplementation levels and amounts and its effect on synovial fluid FA composition is warranted.

Although limited effects of maternal n-3 supplementation on foal bone metabolism markers was observed in healthy foals suckling dams fed to meet NRC requirements, further research is necessary to elucidate n-3 supplementation's potential role as a mediator for DODs.

Future areas of investigation could include n-3 supplementation effects on foals fed to exceed their requirements and grow at an accelerated rate or those predisposed to certain orthopedic diseases, either by genetics or injury. Although no supplementation effects were seen in healthy foals, it is possible n-3 supplementation could provide a protective effect when DODs were induced or bone metabolism was disrupted. Other potential areas for investigation include alternate traits and/or diagnostic techniques. In humans, BMD is an important indicator for bone strength, particularly when orthopedic diseases such as osteoarthritis (OA) and osteoporosis are present (Kaveh et al., 2010). In horses, increasing BMD has been positively correlated with a decrease in orthopedic disease and injury (Kobayashi et al., 2007). In foals, BMD rapidly increases until 2 yr at which time it plateaus, mirroring a normal growth curve (Yamada et al., 2015). In other species, research has indicated that n-3 supplementation can affect BMD (Tarleton et al., 2013) and measurement of this trait may provide a better picture of supplemental n-3 effect on foal bone health. Several diagnostic procedures have been investigated to evaluate BMD in horses, including ultrasound (Jeffcott and McCartney, 1985), x-ray (Bowen et al., 2013), dual energy x-ray absorptiometry (DXA; Vaccaro et al., 2012) and computerized tomography (CT; Waite et al., 2000). The DXA and CT analyses are the most widely used and reliable indicators of orthopedic disease in humans; however, these procedures require access to specialized, expensive equipment in the horse. Although x-ray and ultrasound are relatively inexpensive and accessible, measurements from these techniques are more variable and provide less beneficial data as short-term changes cannot be observed using these procedures. Further research on the effects of n-3 supplementation to a growing foal, with and without DODs, on BMD as measured by DXA or CT could provide a more precise picture of the impact on foal bone health.

Conclusions

Supplementation of marine-derived EPA and DHA during late gestation and early lactation altered plasma FA concentrations in the mare, as previously reported, and also alters the plasma FA concentrations of the *in utero* and suckling foal. Mare gestation length, interval to first postpartum ovulation, and diameter of the dominant follicle at ovulation were not affected by n-3 supplementation. Minimal effect, as evidenced by increased SF ICTP in FS foals, was observed on bone metabolism markers in healthy foals suckling dams fed to meet their NRC requirements. A potential role for supplemental n-3 FAs to improve bone health traits in foals experiencing an orthopedic insult cannot be eliminated by the results and warrants further investigation as a possible mediator for DODs in the foal.

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